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An Identification of TGF-B's Role in Ischemic Cholangiopathy

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by

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LIST OF ABBREVIATIONS

DBD - Donation after Brain Death

DCD - Donation after Circulatory Death

ITBL – Ischemic-Type Biliary Lesions

IC - Ischemic Cholangiopathy

EMT – Epithelial to Mesenchymal Transition

TA/IF – Chronic Allograft Tubular Atrophy/ Interstitial Fibrosis

TGF-β - Transforming Growth Factor-β

BCA – Bicinchoninic Acid

RT-PCR – Reverse Transcription Polymerase Chain Reaction

ICC – Immunocytochemistry

CK-7 - Cytokeratin-7

ABSTRACT

Organ transplants are a necessary intervention for many diseases that result in end stage organ failure. The donation pool cannot match the demands of the transplant list, so expanding the pool to include donation after cardiac death (DCD) is desired. However, there are increased odds of graft failure and ischemic cholangiopathy leading to inferior outcomes when DCD livers are used. Ischemic cholangiopathy consists of multiple diffuse strictures and fibrosis of the bile ducts leading to a loss of epithelialization and fibrosis. The cellular mechanism is proposed to be epithelial-to-mesenchymal transition (EMT). TGF-β is seen as a key initiator of EMT, especially following ischemic reperfusion injury. To determine if TGF-β is an inducer of EMT, cell migration assays were performed with human cholangiocytes exposed to warm and cold ischemia (DCD conditions) and tested for TGF-β expression using western blots, RT-PCR and immunocytochemistry. An inhibitor of TGF-β was also used to show causation. The human cholangiocytes displayed migratory behavior following exposure to DCD conditions as well as an increase in TGF-β expression. Cell morphology also transitioned with a loss of epithelial, cuboidal, characteristics and a gain of mesenchymal, spindle shaped, characteristics. Prior exposure to the TGF-β receptor antagonist prevented increased migration of the cells and retention of the epithelial appearance. Our findings indicate TGF-β plays a major and causative role in the transition of cholangiocytes into mesenchymal cells.

INTRODUCTION

Organ Donation

Organ transplantation is the preferred and highly effective treatment for most patients suffering from end stage organ failure. The benefits come in improved survival and quality of life in patients who receive transplants.[1] However, the number of patients needing transplants outnumbers the amount available leading to a continual shortage of organs for transplant. Because of this shortage, the rate of liver transplant from deceased donors was on average 54.5 per 100 years wait time in the United States in 2018. This results in high rates of mortality in pre-liver transplant patients at 11.8% over a three-year period and a number of patients being removed because they became too sick to transplant.[2] This has led to an increased effort to expand the donor pool and improve outcomes across the board. Options being considered range from improving efficacy of organ preservation solutions and conditions at time of transplant, reducing the number of organs discarded and looking outside the standard pool of organ transplants from brain dead donors and living donors.[3]

Organ Donor Types

The categories for organ donation are living donor donation or deceased donor donation. The deceased donor donation refers to donations of organs where the donor is no longer living, there are two categories with the most common being a donor having suffered brain death and referred to as donation after brain death (DBD). The second category under deceased donor donation is donation after cardiac death (DCD). The difference between these categories lies in state of the circulatory system and viability of the brain; while irreparable damage may have occurred in DCD patients, it may not meet the criteria for brain death.[3] Because the heart must

stop and the circulatory system arrest in the DCD organs, there is a higher risk of damage and complications. DCD livers undergo two periods of warm ischemia: the first is the time between the donor heart stopping and the liver being flushed with cold preservation solution, the second is during implantation of the organ before it is perfused. Reducing the initial ischemic period and developing methods and solutions to help this process are key in reducing damage due to ischemic injury.[3] Even so, there is a more careful selection of DCD grafts compared to DBD grafts as seen by the organ discard rate at 30% in DCD livers donated compared to 6.4% in DBD livers.[1] Though there is a more careful selection of organs in DCD, the rates of graft survival are significantly lower than in DBD livers as well as increased complications.

Patients with DCD livers initially show similar complication rates in the Comprehensive Complication Index (CCI) but at the six-month postoperative mark, there was a significantly higher CCI score for DCD livers compared to DBD livers. For the DCD livers there was a three-fold increase in rates of graft failure due to ischemic-type biliary lesions (ITBL).[4,5] Graft failure resulted from higher rates of early allograft dysfunctions, acute renal failure and severe biliary complications.[5] The rates are increased in this liver pool due to the increased warm ischemia time, transplants with greater than 35 minutes of warm ischemia lead to significantly increased graft failure rates.[6] Complications in liver transplants range from biliary leaks and strictures to ischemic-type biliary lesions. ITBL is characterized by intrahepatic strictures and dilations following ischemic insult.[7] The hypoxic period of the warm ischemia time and hypoxia-generated reactive oxygen species are involved in initiating signaling pathways along with pro-fibrotic cytokines that cause problems after the reperfusion of the organ.[8,9] Cellular injury occurs in part because of the large amounts of oxygen suddenly available again and overwhelming the pathways to scavenge

oxygen free-radicals.[9,10] These cellular mechanisms can help lead to the aforementioned biliary lesions and graft failure, including a disease process termed ischemic cholangiopathy (IC).[10]

Ischemic Cholangiopathy

Ischemic Cholangiopathy is characterized as a set of disorders including multiple diffuse intra-hepatic strictures or non-anastomotic.[10] These strictures occur without any observable cause on imaging studies including a patent hepatic artery or exclusion of a hepatic artery thrombosis. The symptoms generally do not start right after transplantation but are progressive in nature. IC will present the majority of its symptoms between 1 and 6 months and begin with general non-specific symptoms or even asymptomatic in the initial stages and only being diagnosed when liver tests show elevated levels of serum alkaline phosphatase and gamma-glutamyl transferase.[10,11] The progression of the disease will lead to cholestasis and cholangitis and later symptoms of liver failure such as jaundice and itching. This cholestasis can then cause gallstones, biliary sludge and casts and lead to obstruction and cholangitis.[12] The diagnosis of this condition is done by the abnormal liver tests and visual confirmation of the intra-hepatic strictures in the liver by magnetic resonance imaging or endoscopic retrograde cholangiography (Figure 1).[10,13]

Fig 1. Ischemic Cholangiopathy

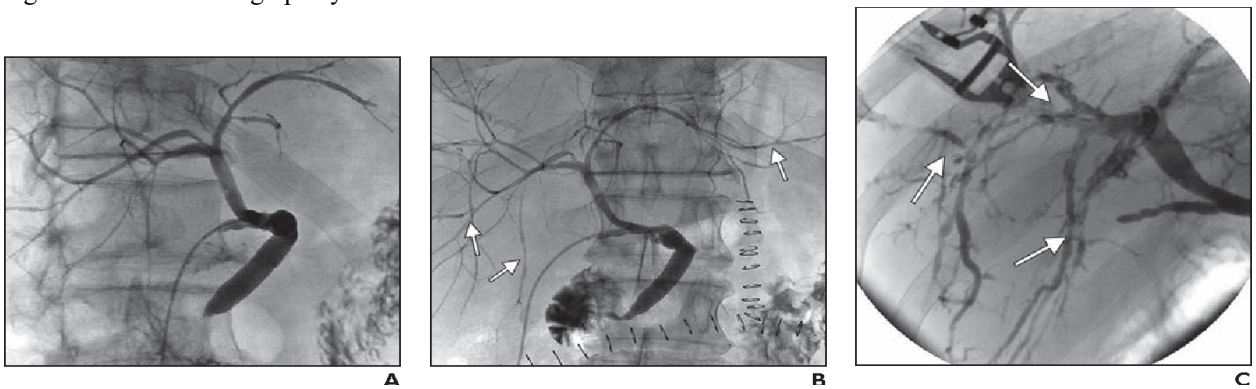

Figure 1: Ischemic Cholangiopathy seen in the cholangiography of the bile duct (including intra-hepatic) of a 59yo male; from left to right the arrows show multiple progressing stenoses after a DCD transplant[13]

Due to the severity of these symptoms, patients diagnosed with IC may have higher readmission rates, lengths of stay, procedures to alleviate symptoms and ultimately 3.2 times higher rate of re-transplantation in DCD livers.[14,15] The rates of ischemic cholangiopathy in DCD livers are between 16% and 29% in comparison to DBD livers between 3% and 17%.[10,15-17] All of this leads to greatly decreased quality of life and is a major problem in using DCD livers.

Due to the severity of this disease and the problems it causes, much research has been put into the underlying mechanisms and methods to prevent it. Ischemic cholangiopathy has three established mechanisms of action: ischemia reperfusion injury, immune response and cytotoxic injury from bile salt toxicity.[18-20] These mechanisms overlap and influence each other, causing the fibrosis of the epithelium and strictures seen in IC. The ischemic reperfusion injury is based on the reoxygenation of the epithelium instead of the anoxic period itself as there is a lower capacity of the cholangiocytes to be able to handle it. The biliary epithelium suffers a high rate of toxic oxygen species formation as stated above that is made worse by low basal levels of glutathione that would help combat these species.[21] The ischemic injury can cause damage to the cholangiocytes as well as to the peribiliary vascular plexus (blood supply surrounding the intra-hepatic bile ducts) than can also damage the cholangiocytes or biliary epithelium and grows worse as cold ischemia time increases.[19]

All these factors play a role in IC and therefore are aimed at preventing its occurrence. Studies have shown that reducing the amount of time organs undergo cold and warm ischemia helps reduce the occurrence of IC as well as using a thrombolytic flush on the liver graft to reduce biliary complications.[22-25] The last technique heavily utilized in preventing the occurrence of IC is using perfusion to optimize the conditions at transplant. IC can be alleviated with low viscosity perfusion of the hepatic artery and peribiliary capillary plexus before transplant. There has been

debate between the comparative efficacies of machine hypothermic or normothermic perfusion with the former showing decreased graft injury rate compared to regular cold storage conditions and normothermic is showing promising reductions in IC as well that need further testing.[26-29] Regardless of these optimizations at time of transplant, the occurrence of graft injury and IC still has a high enough occurrence to warrant a more in depth look at the specific mechanisms of IC.

Epithelial to Mesenchymal Transition

Epithelial to mesenchymal transition (EMT) is a cellular process that results in a loss of epithelium and gain of tissue fibrosis. During this time frame, the cells lose their specific epithelial traits and gain mesenchymal traits and appearance. The cells lose their adhesion and cell polarity in order to dissociate from the epithelial layer and migrate through the extracellular matrix. The newly changed cells gain mesenchymal markers and a fibroblast shape to accomplish the migration.[30] There are three general causes of EMT, the first being what occurs during embryogenesis and the second being what occurs in metastasis in cancer enabling the tumors to travel throughout the body. The last cause of EMT is to escape various stressors such as mechanical, injury repair or hypoxia.[31,32] EMT is becoming a higher focus in various diseases in the liver; it has been observed in fibrotic diseases such as biliary atresia and primary sclerosing cholangitis.[33,34] It has also been observed in a very similar process to IC called chronic allograft tubular atrophy/interstitial fibrosis (TA/IF). TA/IF describes chronic allograft dysfunction in the kidney where EMT also occurs stimulated by myofibroblasts.[36,37]

This prompted the examination of EMT being the mechanism underlying IC. Our lab did an examination of this and looked at morphological changes after ischemia and changes of cellular markers for epithelial and mesenchymal cells. What they found was that in cells exposed to DCD

ischemic conditions, there was a change in morphology from cuboidal to spindle shaped. There was also data collected on the change of expression of both epithelial markers (CK-7, E-Cadherin) and mesenchymal markers (SNAIL, N-Cadherin, Vimentin) by analyzing immunocytochemistry (ICC) results (Figure 2A,B).[37] These results showed EMT serving a role in IC, although certain limitations apply as just cholangiocytes were used. This could miss interactions with other cell populations, such as macrophages, in initiating EMT and producing one of the main inducers of EMT, TGF-β.[38]

Fig2A. Change in Marker Expression

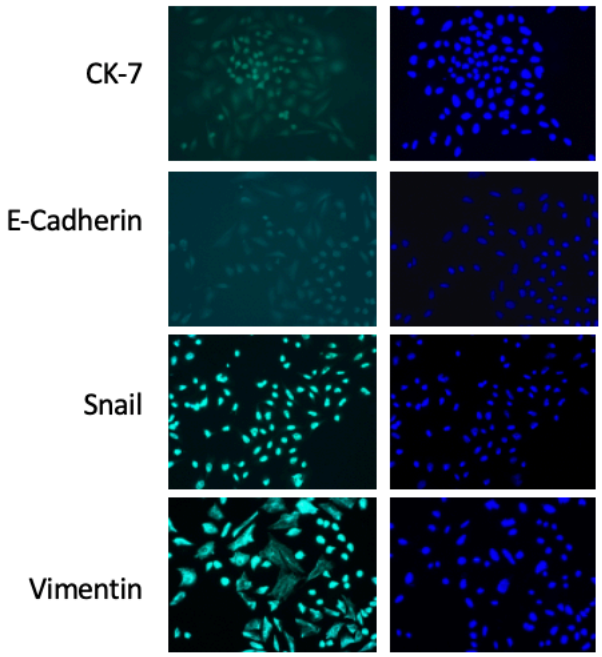

Figure 2A: Immunocytochemistry fluorescence images for the previously done EMT experiments. Results show fluorescence staining of epithelial markers (CK-7, E-Cadherin) and mesenchymal markers (Snail, Vimentin)[37]

Fig2B. Change in Marker Expression

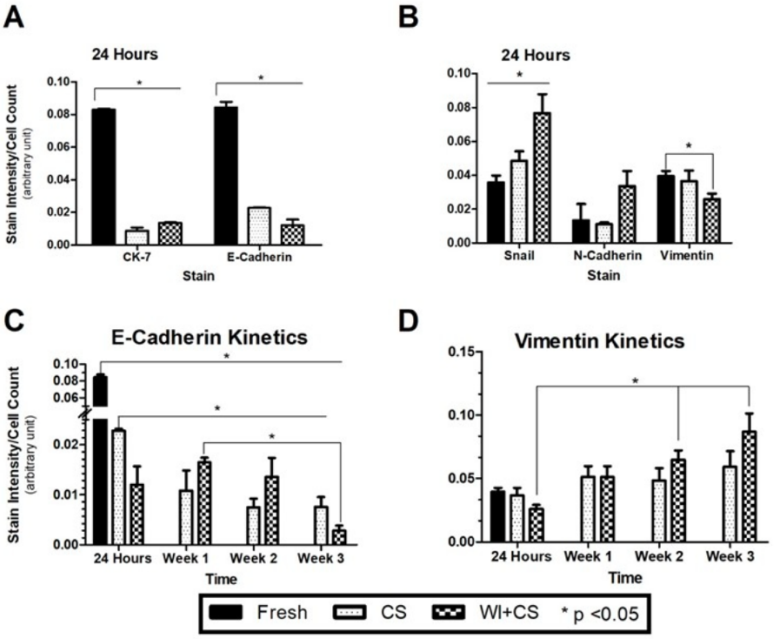

Figure 2B: Immunocytochemical expression of epithelial cell markers CK-7 and E-Cadherin (panel A) and mesenchymal cell markers SNAIL, N-Cadherin, Vimentin (panel B), comparison of fresh cholangiocytes, 24hr after cold storage (CS) and 24hr after 60min warm ischemia (WI+CS). The kinetics of expression of E-Cadherin (panel C) and Vimentin (panel D) are also shown.[37]

TGF-β role in EMT

In order to study EMT more in depth and provide a possible route to reduce IC occurrence, the signaling pathway of EMT needed to be better studied. TGF-β is considered a main inducer of EMT fibroblast activation that leads to tissue fibrosis.[30] This has been established by adding TGF-β to various cell cultures causing a change in morphology and EMT to occur as seen by the change in epithelial/mesenchymal markers.[34] In addition to inducing EMT, increases in TGF-β are seen in cell populations that have undergone EMT as seen in alveolar epithelial cells. To show causation and identify methods of preventing fibrosis, inhibitors such as FGF-1 have been used to prevent EMT and associated marker changes.[30,39] The TGF-β signaling pathway has been studied for EMT, it has been shown that mechanical signaling and growth factor signaling play a role, likely

via proteins in the extracellular matrix.[40] The pathway of TGF-β interaction likely includes SMAD phosphorylation once TGF-β binds its receptor via paracrine signaling. This pathway leads to other transcription factors and families of proteins causing the necessary EMT changes (Figure 3).[41]

Fig3. TGF-β signaling pathway

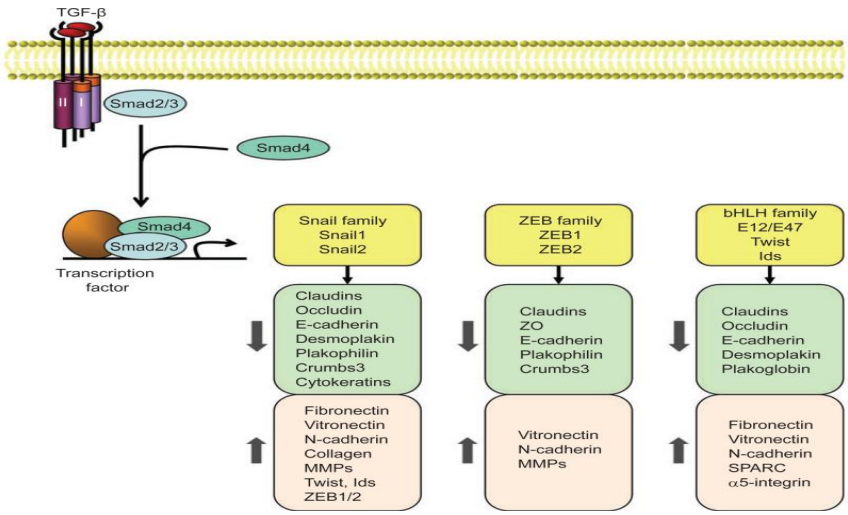

Figure 3: TGF-β signaling pathway that induces EMT, including transcriptional regulation. After TGF-β binds the receptor, SMAD2/3 form complexes with SMAD4 to regulate transcription of the target genes via three different families of transcription factors. Ultimately epithelial marker gene expression decreases while mesenchymal increase.

Purpose of Study

The purpose of this study is to identify a potential role of TGF-β in epithelial to mesenchymal transition seen in ischemic cholangiopathy. Previous studies in the lab showed EMT serving a role in the loss of cholangiocytes during DCD organ transplant conditions.[37] To further investigate these findings, this study sought to determine if TGF-β expression rose with ischemic conditions simulating DCD organ transplant. The second part was to use an inhibitor of TGF-βR1 to try and prevent EMT from occurring. TGF-β was chosen since it has been shown to be a potential initiator of EMT and we are interested in determining the initiating mediator in our models of IC.

The study is replicating the effects of DCD conditions with a period of warm ischemia followed by cold storage ischemia and then recovery and reperfusion on cultured primary human cholangiocyte cells. By analyzing changes in TGF-β expression and cell morphology, we were able to determine if TGF-β served a correlative role in the epithelial to mesenchymal transition seen in ischemic DCD conditions. All of these were measured in vitro so the possible interactions with other cell populations, that may serve as a source of TGF-β, are not seen. However, the impact of this study could be far reaching as an alternative pathway to reduce ischemic cholangiopathy occurrence and expand the available donor pool.

METHODS AND MATERIALS

Human Cholangiocyte Cells

Primary human cholangiocytes cells (HCC), biliary epithelium, (Celprogen, cat.#36755-12) were cultured in flasks using human cholangiocyte cell growth medium with pre-added serum and antibiotics (Celprogen, cat.#M36755-12S) and grown in an incubator with 5% CO_2 at 37°C. Cells were grown to a maximum 80% confluence for use and kept within eight passages. Trypsin (0.05%, Quality Biological) was used to passage the cells. The centrifuge was used to spin cells down at various points for 5 minutes at 1000 rpm. Other media used during experiments was a 50% DMEM (Gibco) with added fetal bovine serum (10%, Thermo Fisher) and antibiotic antimycotic solution (1%, Gibco) and 50% HCC media for the mesenchymal cells after ischemia storage. Cell counts for various aspects of the experiments were done with a hemocytometer and light microscope at 10x magnification. The ratio of trypan blue to cells in media was 4:1 for the cell counts.

In the basic cell model to simulate DCD conditions, cells are cultured for at least 24 hours pre-experiment to ensure sufficient attachment to the plates. Next, the cell cultures are placed in airtight (Tupperware) containers that undergo purging of the box atmosphere with 95% nitrogen and 5% CO_2 for 10 minutes and then set in the incubator for 60 minutes to simulate the warm ischemia period between cardiac death and organ flushed with cold preservation solution. After this the container is placed in a larger airtight container in a layer of melting ice. The atmosphere is purged again, and the large container is placed in a fridge kept at 4°C for 24 hours to simulate cold storage and preservation of organs. After storage, the cells are removed from the containers and exposed to atmospheric oxygen again then placed back in the incubator and cultured as normal, this simulates reperfusion. After reperfusion the cells were collected at 1 day (for control and

ischemic conditions), 5 days and 7 days. The cell populations for the basic ischemic model are fresh control cholangiocytes (CC) without ischemia, ischemic samples collected at day 1, day 5 and day 7.

Cell Migration Assay

To separate cell populations that undergo migration after ischemia, Cytoselect invasion chambers (Cell Biolabs, cat.#CBA-110-COL) coated with type 1 collagen were used in conjunction with ischemia (Figure 4). The chambers were composed of wells with a collagen matrix sitting on top of a nylon filter. There were 300,000 human cholangiocyte cells placed in each well in 0.3mL of human cholangiocyte media. Each well was placed in a 24-well plate and a 0.8mL mixture of 50% human cholangiocyte media and 50% DMEM was placed on the outside of each well. After being placed on the chambers they were allowed to culture for 24 hours. After this the cultures were subjected to ischemia representing DCD conditions as described above.

Fig4. Cell Migration Assay

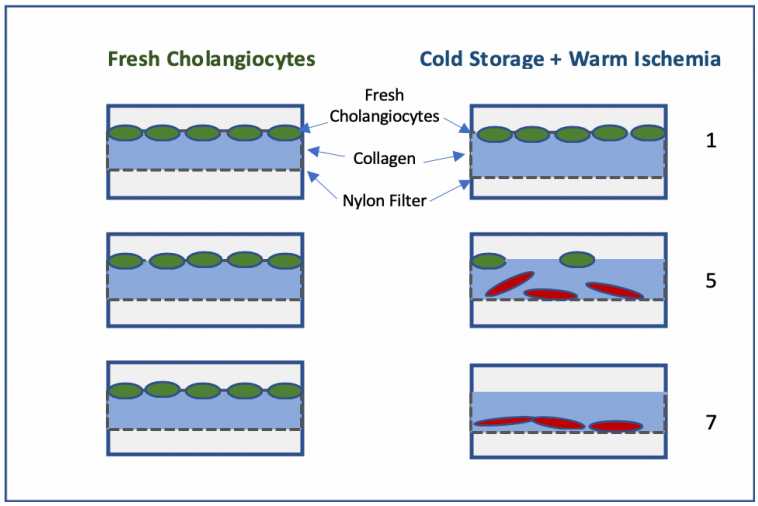

Figure 4: Cell Migration Assay to study migratory behavior after being exposed to DCD conditions over 7 days. Fresh cholangiocytes (left) remain on top of the collagen gel matrix with no migration. On the right, cholangiocytes exposed to 1hr warm ischemia and 24hr cold storage migrate through the collagen matrix to rest on the nylon filter below.

After the cells are removed from the storage ischemia, they are placed back in the incubator for varying timeframes to allow time for migration. The three different timepoints used for collection after cold storage ended were the same as above, 1 day, 5 days and 7 days. The initial experiment with these chambers was 3 days instead of 5 days. During these time frames media was changed every three days with inhibitor also being added again. Control populations that did not undergo ischemia also were used and pulled at the same timepoint as the 1-day culture. There were two different cell populations after the timepoints were finished, the cells that remain on top of the collagen matrix functionally resembling epithelial cells still. The cells at the bottom of the collagen matrix on the nylon filter and on the bottom of the plate functionally resembling mesenchymal cells. To recover the top culture, a cotton swab was gently run over the surface of the collagen and swirled in a tube with Phosphate Buffered Saline (PBS). Next the PBS tube was centrifuged for 5 minutes at 1000 rpm. The PBS is then aspirated off the top and trypsin is mixed in for 5 minutes at which point media is added and centrifuged down again. The trypsin/media is aspirated and 1mL of media is added, 10uL of this mixture is mixed with 40uL of trypan blue and a cell count is performed.

For the bottom cell population and after the top layer is removed trypsin is applied to the nylon filter and bottom of the plate for 5 minutes. After this media is added and this mixture is moved to a tube and centrifuged down. Next, the trypsin/media is aspirated off and 1mL of media is added with 10uL removed and added to 40uL of trypan blue and a cell count is performed. When the cell count is finished the cells in the media are added to fresh wells to culture for further analysis via western blot and reverse transcriptase polymerase chain reaction (RT-PCR). The cell samples collected are cholangiocyte control (CC) without an invasion chamber, control top/epithelial (CE) and bottom/mesenchymal (CM) without ischemia, top (DE) and bottom (DM) days 1, 5, and 7.

TGF-β Receptor Antagonist

To determine if TGF-β serves a causative role, an inhibitor was used. Galunisertib (Tocris, Cat.# 6956) is a TGF-βR1 inhibitor with an IC_{50} value of 172nM and molecular weight of 369. It also inhibits TGF-βR2 with sub-micromolar IC_{50}s. The inhibitor was solubilized in DMSO to create a stock 1 solution inhibitor concentration of 5ug/uL in DMSO. A stock 2 solution was made by adding stock 1 to HCC media at a 1:100 dilution for an inhibitor concentration of 0.05ug/uL in HCC media. The concentration of inhibitor applied to the cell samples was then 1uM. A vehicle control was also performed using the same concentration of DMSO without inhibitor mixed in HCC media. After the inhibitor was added, there was an hour between the time of application and time of warm ischemia to allow the inhibitor time to work. Ischemic conditions were applied as described above. After the cold storage ischemia was complete the cells were kept for analysis in the same way as the non-inhibitor cell populations. The inhibitor was used in the cell morphology and immunocytochemistry (ICC) experiment. The cell populations used were the control group consisting of a fresh cholangiocyte control (CC), vehicle DMSO control (CD), inhibitor control without ischemia (CG), the ischemic group (D1, D5, D7) and inhibitor group (GD1, GD5, GD7).

Western Blot

To collect protein, cell cultures were washed with PBS then given 0.5mL of RIPA buffer and 0.05mL of proteinase inhibitor. The samples used were collected after the cell migration assay at the specified timepoints and included fresh cholangiocytes. After this, a Bicinchoninic Acid Assay (BCA) was performed with an albumin standard curve. This curve was used to determine the protein concentration with a Biotek microplate reader. Based on the concentration, the samples were denatured and reduced with added Laemmli buffer and 2-Mercaptoethanol and boiling the

samples for protein concentrations of 50ug/20uL per well. Samples were stored in the -20°C freezer until use.

Electrophoresis was performed by loading 20uL of sample per well alongside 10uL of the ladder and run on Bio-Rad precast gels and SDS-PAGE (10%) with 1xMOPS SDS running buffer. The gels were run at 50V for 30 minutes and 150V for the remainder of the run until the loading dye reaches the bottom of the gel. The protein was then transferred onto Polyvinylidene fluoride (PVDF) with Novex transfer buffer. The PVDF membranes were first soaked in 100% MeOH and then equilibrated in transfer buffer alongside gel membranes. Filter pads are placed on each side of the membranes and fiber pads on the outside of these. This transfer was run for 60min at 100V. After transfer the blots were soaked in TBS/Blotto-B with 0.25% Tween-20 to block non-specific binding sites. After blocking, the membranes are incubated with 1:500 concentration of primary TGF-β antibody in the same Blotto-B solution. This was done for 18 hours on an orbital shaker at 4°C. There was trouble using the loading control protein, GAPDH, that has a concentration of 1:1000 concentration of primary/secondary antibody. After this period the blots were washed with multiple fast rinses of water followed by two washes of Blotto-B for 5 minutes each on an orbital shaker. The secondary antibody is then added conjugated to HRP at a 1:1000 dilution for 60 minutes at room temperature. Another wash is performed using fast rinses of water, two washes with 0.05% Tween-20 in TBS for 5min followed by two washes with 0.1% Tween-20 in TBS for 5min each and then a wash in 0.5M NaCl in TBS for two minutes. The blots were then developed in 15mL Santa Cruz Luminol reagent for 5min at room temperature and imaged for chemiluminescence on a ChemiDoc. ImageJ was then used for analysis.

Reverse Transcription Polymerase Chain Reaction

To study gene expression, RT-PCR was performed. The samples used were collected after the cell migration assay at the specified timepoints and included fresh cholangiocytes, though issues with storage prevented same experiment comparison. The bottom cell samples were from an earlier experiment and included 1 day, 3 day and 7 day. Cells were washed with PBS before beginning RNA isolation using the lysis Buffer RLT with added 2-Mercaptoethanol. This homogenized lysate was then put through a DNA spin column and then RNA spin column that is washed multiple time to isolate and purify the RNA. This purified sample is then measured for concentration using the Nanodrop One. This gave concentrations (ng/uL) and ratios of A260/280 and A260/230 that indicate RNA purity and sample purity. These were checked to ensure the samples were sufficient to undergo RT-PCR. After this, the values were input into cDNA synthesis protocol calculation to determine amounts of reverse transcriptase, nuclease-free water and sample to add. Next the samples were put into a thermal cycler; the priming stage was 5min at 25°C, reverse transcriptase was 20min and 46°C and finally RT inactivation for 1min at 95°C. The samples were then held at 4°C.

Once this was done, the samples underwent RT-PCR with the specific primer sequences added. TGF-β was added with both the 5'-3' forward primer and the 3'-5' reverse primer as well as double deionized water. GAPDH was also used with the same requisite primer sequences as an internal control or housekeeping gene. Each sample was done in triplicate for both the TGF-β and GAPDH. Once the final data was collected, the samples underwent statistical analyses.

Immunocytochemistry

The cells underwent the basic ischemic model described above without the invasion chambers and included fresh cholangiocytes, control with DMSO, control with inhibitor Galunisertib, 1 day, 5 day and 7 day as well as the corresponding inhibitor days matching each. Cells were cultured and a $5x10^4$ density was grown on the sterilized cover slips before being fixed in 4% paraformaldehyde in PBS. For the morphology pictures, the cell cultures were fixated without cover slips and then imaged with a light microscope. After washing with PBS, the cells were permeabilized with 0.15% TritonX-100 followed by blocking unspecified binding with 5% donkey serum in PBS. After this, the cells were incubated with the primary antibodies for two hours and diluted in 5% donkey serum. The concentration of antibody used for TGF-β and for cytokeratin-7 (CK-7) was 1:100. After this the cells were washed with 0.1% Tween20 in PBS and PBS and then incubated with secondary antibody (rabbit IgG) at a concentration of 1:200 in the dark. Secondary antibody control testing was done without applying the primary antibody. The cells were then washed again before mounting on cover slips. Fluorescent imaging was performed with the Zeiss Axioimager A1 microscope. Microscopy was performed at the VCU Microscopy Facility, supported, in part, by funding from NIH-NCI Cancer Center Support Grant P30 CA016059.

Statistical Analysis

All data were tested for distribution normality. Most data were analyzed by parametric one-way ANOVA with Tukey HSD and Bonferroni multiple comparison correction. The ICC data was also analyzed with t-tests to compare the ischemic group to the ischemic plus inhibitor group. Most data are expressed as mean plus or minus the standard deviation. The analytical experiments were

usually run in duplicate or triplicate. Statistical analysis was performed using ImageJ, Microsoft Excel and Prism software. A P value less than 0.05 was considered statistically significant.

Troubleshooting

The initial cell migration assay was repeated due to failure to collect the top cell layer. Due to COVID-19 and lab renovations, assay samples had to be tossed before the ICC was optimized and RNA samples misplaced, preventing a proper dual experiment. COVID-19 also forced a tight time frame preventing ideal quality of ICC images, it took five rounds of ICC to obtain decent images. Some ICC slides were still unable to be used. Multiple occurrences of bacterial contamination prevented a final alternate inhibitor study and a repeat paired RT-PCR experiment. Western blot loading control also was unable to work, likely due to technical error.

RESULTS

Cell Migration Assay

Cholangiocytes that were put under DCD ischemic conditions in invasion chambers showed greatly increase migratory behavior (Figure 5). The cells were counted after ischemia and a hemocytometer was used in order to average the cells per area. These cells also showed a great decrease in epithelial growth in comparison. The control timepoint represented as zero time, were collected at the same time as the day one recovery but without ischemia and showed some migration as well. The top population is functionally acting as epithelial cells and the bottom population is functionally acting as mesenchymal cells.

Fig5. Cell Count Invasion Chamber

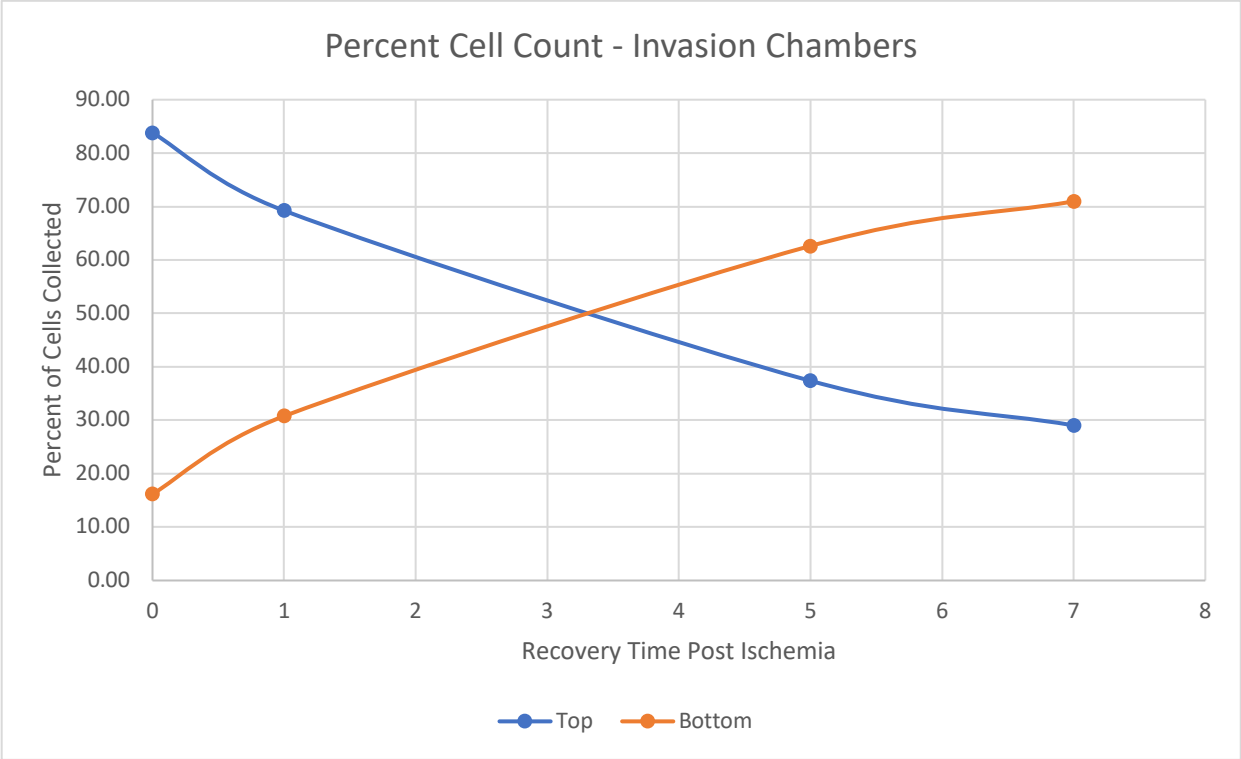

Figure 5: Percent cell counts performed at the different timepoints for the top and bottom cell populations of the invasion chambers. The zero-time point is the control population collected 24hr after culture. Percent refers to taking the specific cell population (top/bottom) and dividing by all cells collected at that timepoint (top+bottom). N = 1

Following the above experiment was another use of the invasion chambers to measure migratory behavior with a TGF-β inhibitor and DCD ischemic conditions. The percent cell counts showed a decrease in bottom or mesenchymal cell population in comparison to the top or epithelial population (Figure 6). There was a decrease in epithelial to mesenchymal transition because of the inhibitor application in comparison to the previous migration assay. The next step was to study the changes in TGF-β expression in these collected cell populations.

Fig6. Cell Count Invasion Chamber with Inhibitor

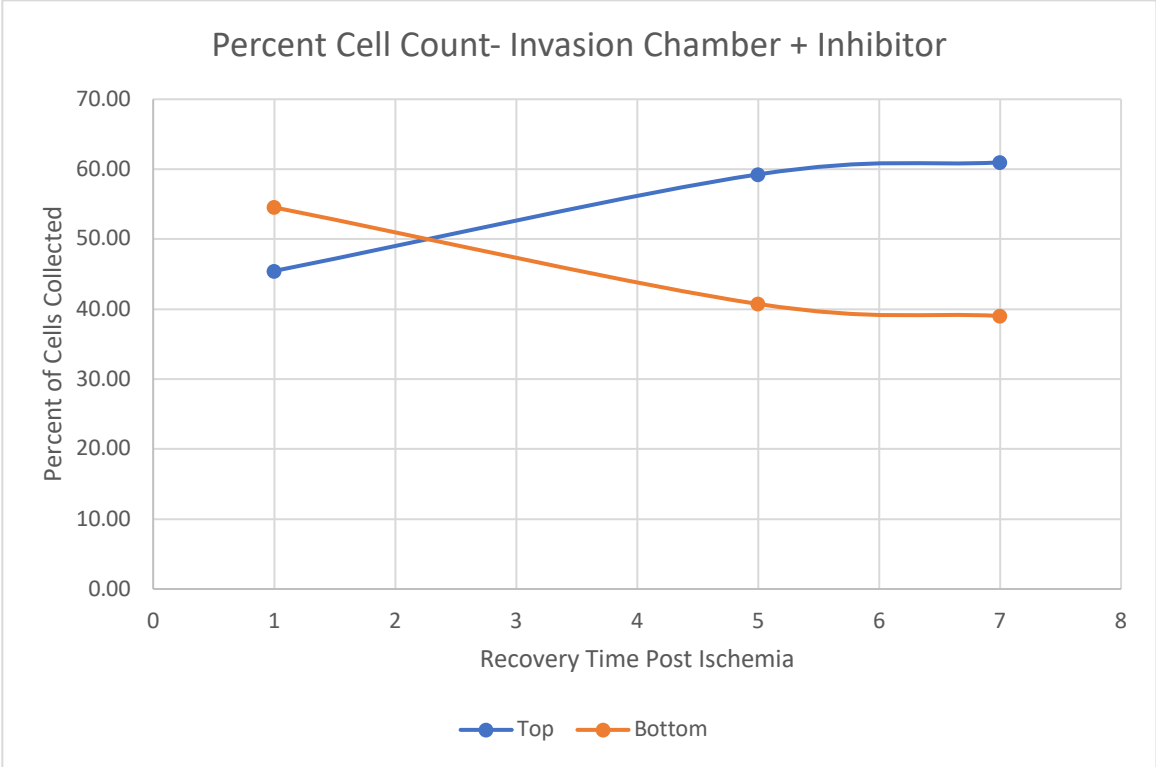

Figure 6: Cell counts performed at the different time points of recovery with inhibitor added into the invasion chambers. These are percent counts and show an increase in the top cell population relative to the bottom. N = 1

TGF-β expression (WB, RT-PCR)

TGF-β expression was then measured by analysis of protein and gene expression. The RT-PCR data showed significant differences in the bottom or mesenchymal cell populations in comparison to the top or epithelial cell populations (Figure 7). All three were significant in comparison to all other samples, the day 7 top was significant in comparison to the control fresh cholangiocyte sample. There was a problem with sample storage that necessitated using previous bottom cell collection, so the bottom has a 3 day while the top has a 5 day.

Fig7. RT-PCR Results

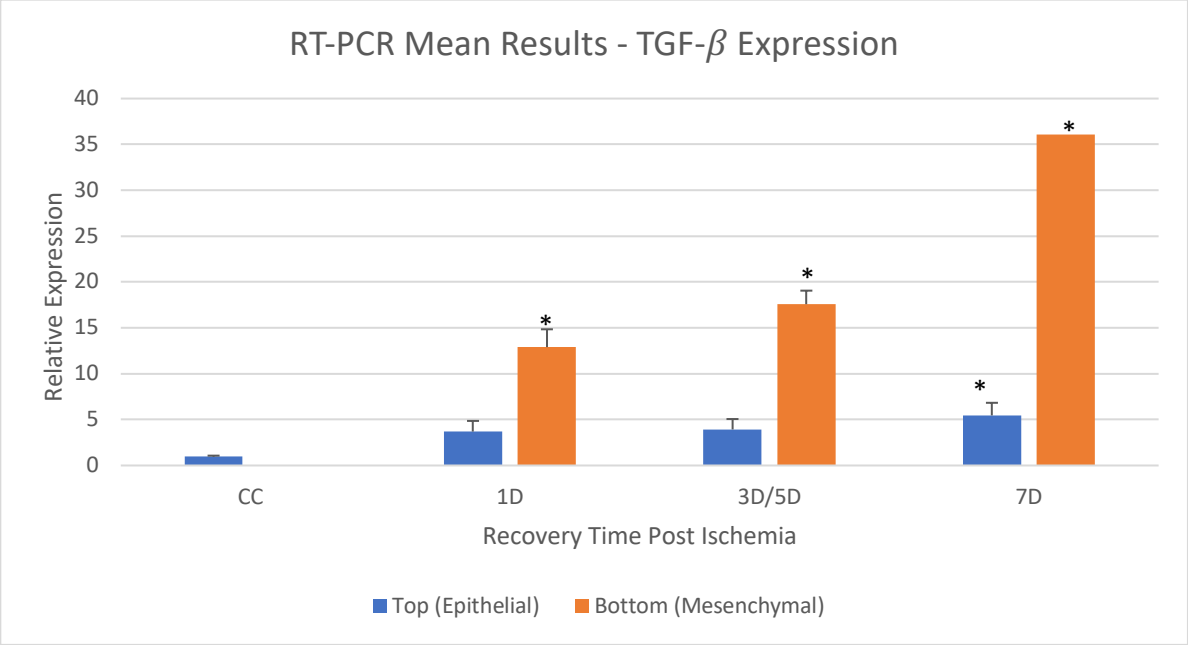

Figure 7: RT-PCR results for the invasion chamber populations on TGF-β gene expression. The CC is fresh control cholangiocytes, top and bottom are the respective top and bottom layer from the invasion chambers at the three time points. Bottom is 3 Day while the top is 5 Day. P value significance is set at less than 0.05 and indicated with an asterisk. The samples are the relative expression measured to the internal control GAPDH. N = 3

The western blot results tested for protein expression of TGF-β in the invasion chamber cell populations collected (Figure 8). The samples were loaded with the same volume and concentration. The results showed a lack of power in order to determine significance. However,

there is a trend seen both visually with the averaged samples from the duplicates and with normalized data to the control that suggests the bottom mesenchymal protein expression of TGF-β increases while the top epithelial protein expression stays about the same as the control cholangiocyte samples (Figure 9). The GAPDH loading control was not able to work.

Fig8. Western Blot

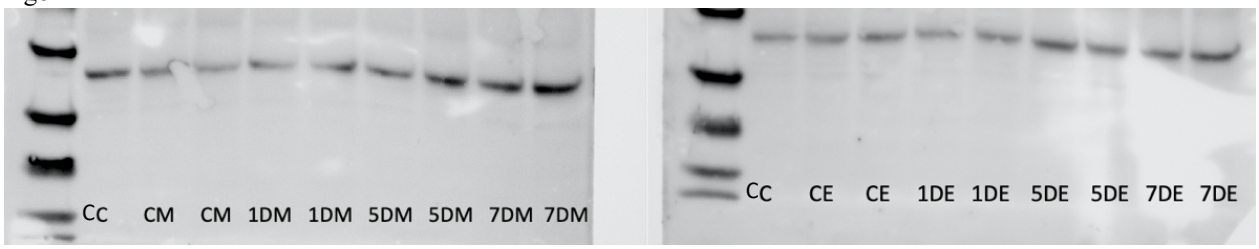

Figure 8: Western Blot looking at protein expression of TGF-β at 44kDa. This was performed with a ladder and in duplicates of samples. Samples include Control (CC), bottom or mesenchymal control (CM) and top or epithelial control (CE) that did not undergo ischemia. The rest of the samples were collected at 1 day, 5 days and 7 days from the top or bottom of the invasion chamber.

Fig9. Western Blot Results

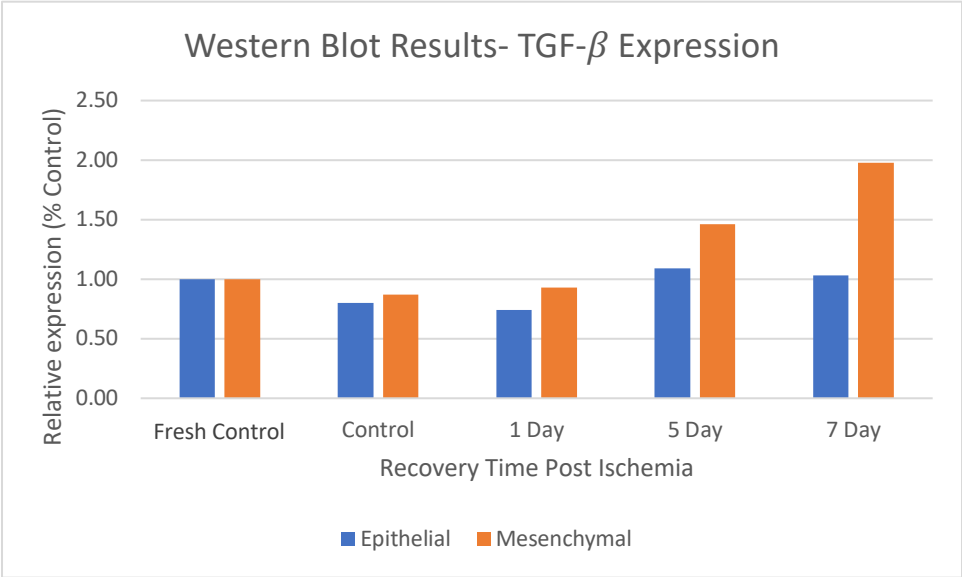

Figure 9: Western Blot results did not show significance due to low power, so the data was normalized to the control. Each value was the average of two loadings on the gel and then divided by the control value. The cell samples are from the top (epithelial) and bottom (mesenchymal) of the invasion chambers at 1, 5 and 7 days. N = 2

Cell morphology

Cell morphology was another important aspect for studying the effects of the inhibitor. The data was calculated by grading the images based on epithelial or mesenchymal characteristics (Figure 10). Grade I is a distinctly epithelial appearance (small, cuboidal), grade II is a mostly epithelial appearance, grade III is a mostly mesenchymal appearance and grade IV is a distinctly mesenchymal appearance (elongated, spindle). Each image was divided into four quadrants and graded. The percent of each grade out of total cells was used for the mean value.

Fig10. Morphology Images

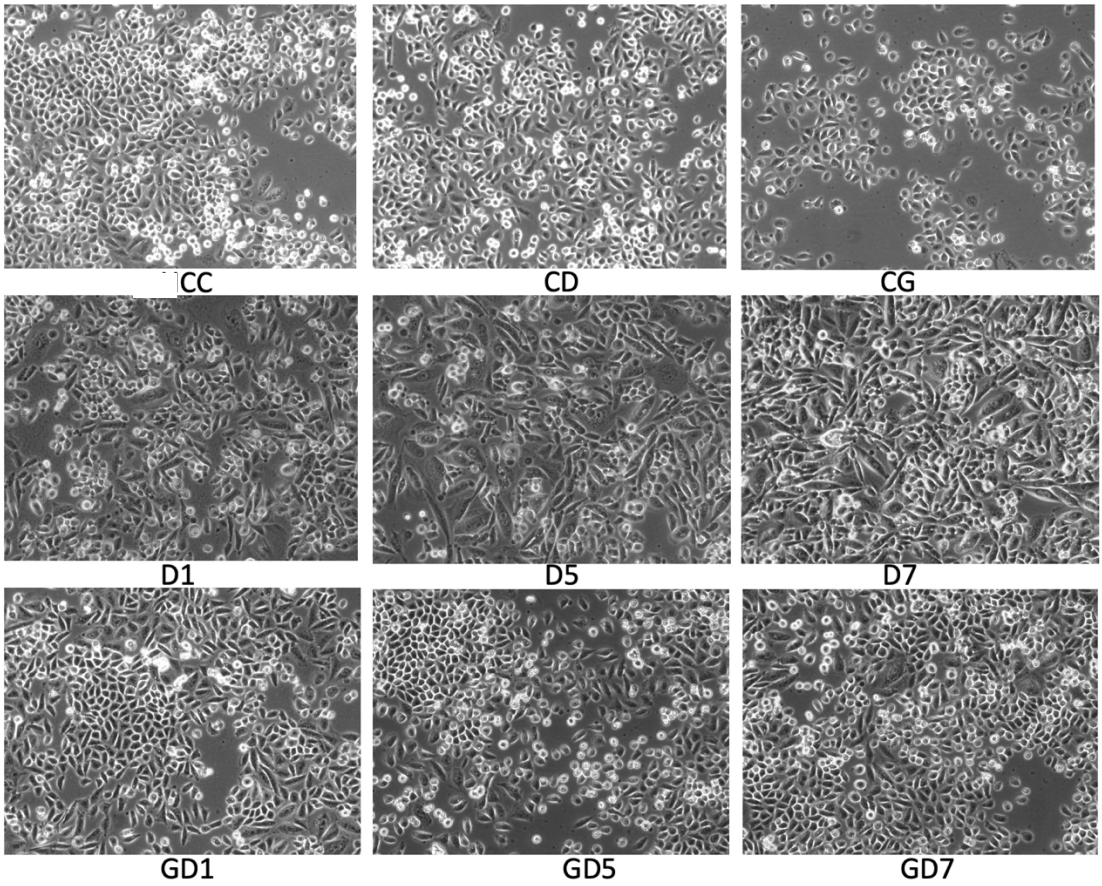

Figure 10: Cell morphology change with exposure to DCD ischemia. CC refers to fresh control cholangiocytes, CD is the DMSO vehicle control for the inhibitor, CG is the inhibitor Galunisertib control (no ischemia), D1,5,7 underwent DCD conditions without inhibitor and GD1,5,7 underwent DCD conditions with inhibitor.

Significance is seen in the cells that underwent ischemic DCD conditions, without an invasion chamber, in comparison to the fresh cholangiocytes and other controls except for the ischemia treated day 1 (D1) in the grade I epithelial category (Figure 11A,B). The inhibitor day 1 (GD1) showed significance in comparison to the fresh cholangiocytes for grades II, III, IV. Significance was also seen between the ischemic D5, D6 samples and the inhibitor GD5, GD7 samples except in grade II.

Fig11A. Cell Morphology Results

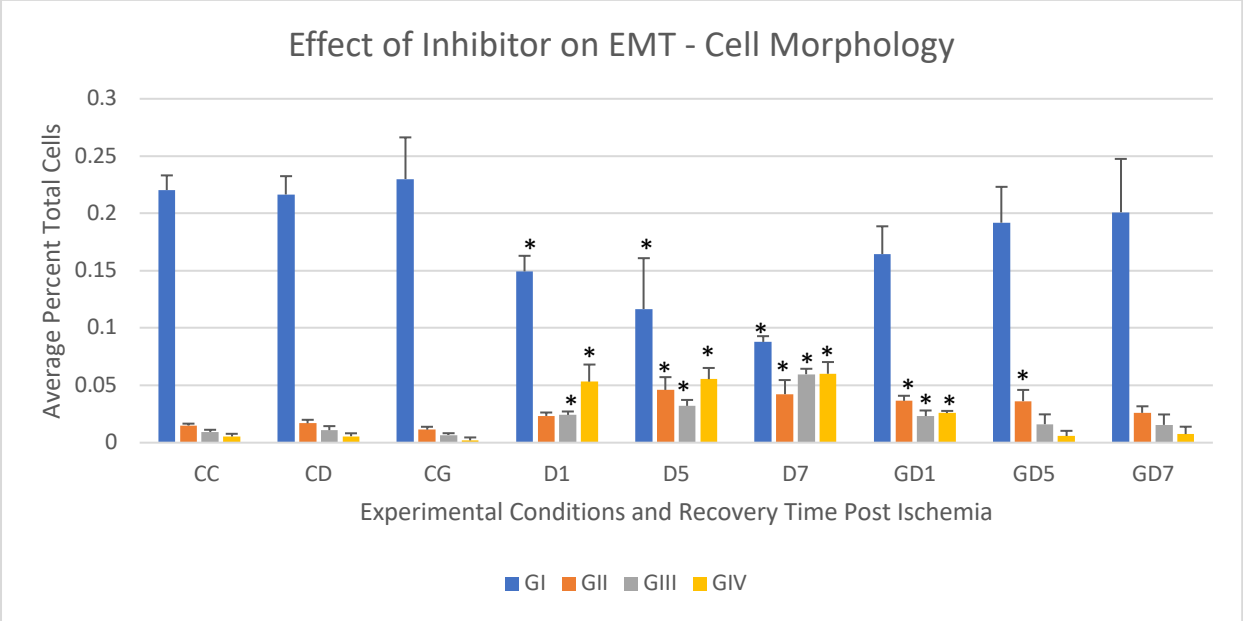

Figure 11A: Cell morphology results from the grading of pictures taken with light microscopy. Data is plotted time points against the mean percent of cells out of total cells. There are three main groups, the control group with fresh cholangiocytes (CC), vehicle DMSO control (CD), and inhibitor control (CG). The second is ischemia treated time points (D1,D5,D7) and third is ischemic conditions plus inhibitor treatment (GD1,GD5,GD7). Significance with P value <0.05 is indicated with an asterisk. G1 refers to cuboidal appearance, GII mostly cuboidal appearance, GIII refers to mostly spindle appearance and GIV refers to spindle appearance. N = 4

Fig11B. Cell Morphology Results- Grading

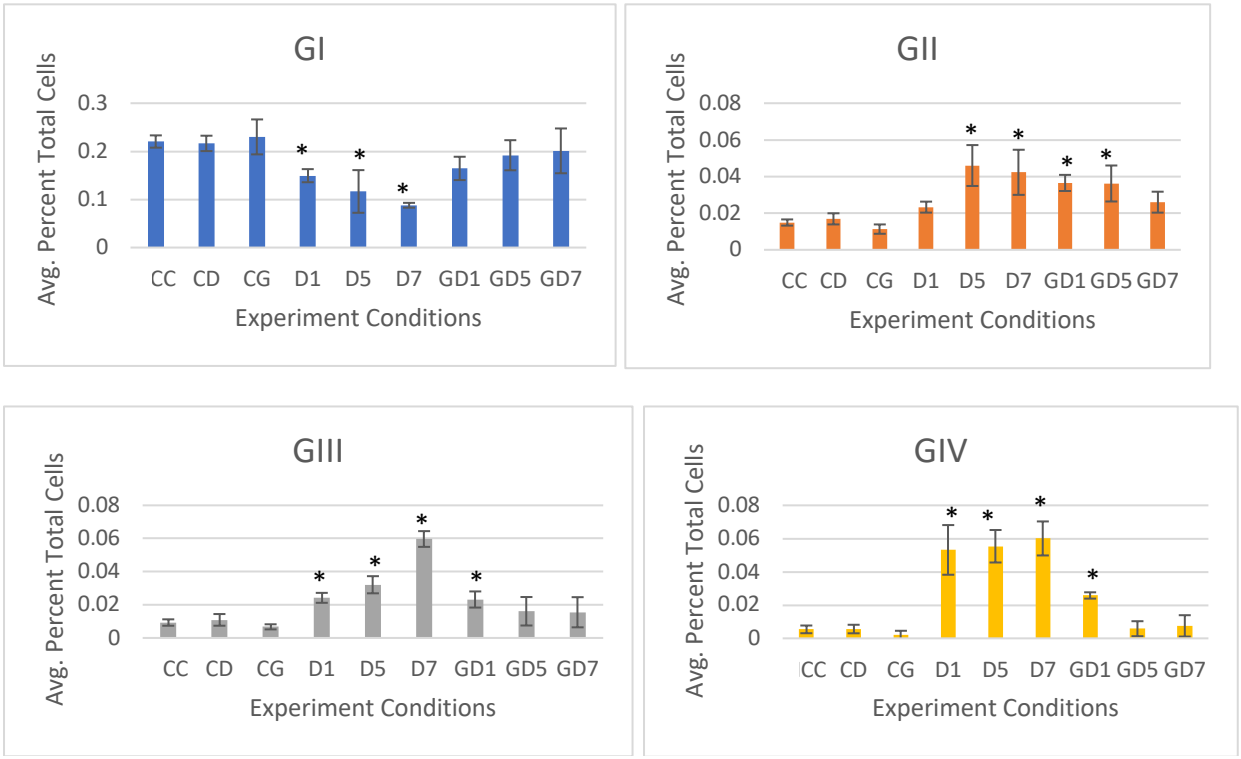

Figure 11B: Cell Morphology results with each of the gradings pulled into their own graph. Grade I (top left) appear distinctly cuboidal (epithelial), grade II (top right) appears mostly cuboidal, grade III (bottom left) appears mostly spindle shaped and grade IV (bottom right) appears distinctly spindle shaped (mesenchymal). The same cell test populations are used as before. P value is again indicated with an asterisk and significance level of less than 0.05. N=4

Immunocytochemistry

After undergoing DCD ischemia, the cells were tested for TGF-β to show changes in concentration (Appendix A). Inhibitor was also applied to these cells. Significance was seen between all samples within the ischemia group and in the ischemia plus inhibitor group all were significant except for control inhibitor and day 7 inhibitor (Figure 12). Statistical significance was also seen between the inhibitor group and the non-inhibitor ischemic group at the comparative timepoints (control to control, D1 to D1, D7 to D7). Secondary antibody controls were also performed to ensure there was no nonspecific binding (Appendix A).

Fig12. ICC Results

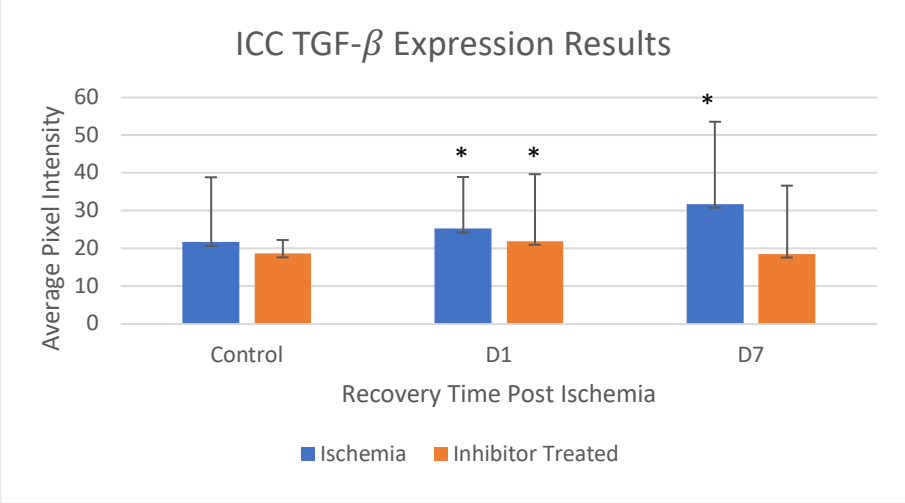

Figure 12: ICC Results for TGF-β, the time points are plotted against the average pixel intensity with comparison between the ischemia plus inhibitor treated and ischemia untreated group along with controls of each that did not undergo ischemia. Control refers to a fresh cholangiocyte control for the ischemia group without ischemia and inhibitor control without ischemia for the inhibitor group. Significant P value of less than 0.05 indicated by asterisk. Ischemia control (N=299), D1 (N=611), D7 (N=588), inhibitor control (N=403), D1 (N=911), D7 (N=1556)

Immunocytochemistry was again performed to see the differences in CK-7, an epithelial cell marker (Appendix A). The groups were again ischemia vs inhibitor treated with a fresh cholangiocyte control for ischemia and an inhibitor control (Figure 13). There was a significant difference detected between all samples within the two groups. There was also a significant difference when comparing across the groups (control to control, D1 to D1 and D7 to D7).

Fig13. ICC CK-7 Results

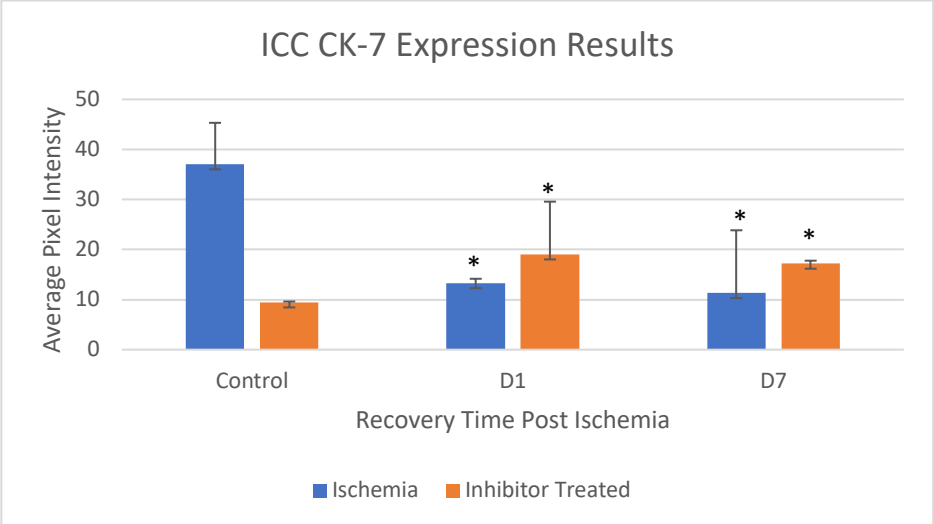

Figure 13: ICC results of CK-7 testing across the inhibitor treated ischemic group and the non-inhibitor treated ischemic group. Control refers to fresh cholangiocytes for the ischemic group and an inhibitor control for the inhibitor group. D1 and D7 refer to Day 1 and Day 7 for both groups. Significant P values of less than 0.05 are indicated by an asterisk. Ischemia control (N=272), D1 (N=493), D7 (N=849), inhibitor control (N=351), D1 (N=647), D7 (N=878)

DISCUSSION

Major Findings

Ischemic Cholangiopathy is a serious condition affecting the bile duct with a high occurrence in DCD livers. The disease can lead to graft failure, need for re-transplantation and higher rates of mortality. Because of this high occurrence in DCD livers, there have been efforts to optimize transplants in these conditions to reduce IC. However, the rates are high enough to warrant further investigation into the exact mechanisms to try and solve the problem a different way. Previously in our lab, they found that when DCD conditions are applied to cholangiocyte cells, they undergo fibrosis and migration, losing their epithelial markers in favor of mesenchymal markers. This epithelial to mesenchymal transition or EMT was identified as a possible mechanism due to the similarity of other ischemic injuries leading to fibrosis. Because EMT was shown to occur in these ischemic conditions, the next step was finding the pathway signaling molecule that can prevent EMT from occurring.

The focus of this research is on the role TGF-β plays in initiating the epithelial to mesenchymal transition that occurs in IC. As such, changes in TGF-β expression were studied and there was a significant increase in TGF-β expression when ischemic DCD conditions were applied, and migration occurred. This trend shows a strong correlation between the EMT occurring and TGF-β. To look more into causation, an antagonist of TGF-βR1 was used. This inhibitor was shown to decrease the migratory behavior of cells exposed to DCD conditions as well as prevent the transition to mesenchymal appearances and retain an epithelial marker. All of this together makes a strong case for TGF-β playing a role in initiating EMT after ischemia in human cholangiocytes. More studies need to be done in order to determine its clinical significance, but it serves as another possible path to reduce ischemic cholangiopathy in DCD liver transplants.

Phenotypic Changes

In order to better study changes in TGF-β expression, invasion chambers were used to both see migratory behavior and select out the populations that transitioned. When DCD conditions were applied to the cholangiocytes, there was a clear increasing trend of migration that indicated EMT was occurring. This trend was an increased rate of cells that migrated down through the chamber compared to the cells that remained on top of the collagen matrix. This necessity to transition through a collagen matrix meant that the cells had to functionally change in order to move through. One thing to note was that, although few, there was a small number of cells that migrated without exposure to ischemia. However, there was a large increase in cells that transitioned with just one day of recovery time after ischemia exposure. The change in morphology that accompanied the transition was seen in both previous experiments done in the lab and the current experiment. There was a significant increase in spindle shaped cells, or mesenchymal cells, in cultures that underwent ischemia separate from the invasion chambers. Other phenotypic changes associated with EMT occurred as well; the lab had previously shown the reduction in epithelial markers (CK-7 and E-cadherin) leading to an increase in mesenchymal markers (Snail, Vimentin). Due to this, only the CK-7 epithelial marker was used in the ICC experiment to determine if epithelial characteristics were retained. The significant difference in the ischemia time points from the control indicates these cells were transitioning from epithelial cells. Overall there were significant phenotypic changes that occurred with DCD conditions that help reveal the role of TGF-β when compared to the TGF-β Receptor inhibitor.

TGF-β expression

After using the invasion chambers, the cell populations were collected in order to test for TGF-β expression. Since the populations were distinguished by functional changes leading to transition through collagen, this allowed for much greater selection to occur. The bottom culture should selectively resemble mesenchymal characteristics and express an increase in TGF-β during transition. The cells remaining in the top of the culture should functionally remain epithelial and therefore not show as much increase in TGF-β expression. This of course was not a perfect experiment as previous invasion chamber studies in the lab showed a maximal migration around day 21, these experiments were all collected at a maximum of day 7. This means there could have been cells still on the top of the chamber that were preparing to functionally change but had not transitioned yet. This would explain slight increases in TGF-β expression in the top cell samples.

TGF-β expression was significantly increased in the RNA collected from the mesenchymal bottom cell population. This indicated that there is a correlation between ischemic induction of EMT and TGF-β. By day 7, there was a very large increase in TGF-β expression that clearly showed this correlation. Western blots were also performed to study the changes in TGF-β protein expression following DCD conditions. Although significance could not be tested due to the semi-quantitative nature and low repetitions, there was a visible trend occurring with an increase in TGF-β expression as time progressed in the mesenchymal cell population. Conversely, in both RT-PCR and western blot, the epithelial cell populations TGF-β expression remained about the same or increase much more slowly which is as we expected.

Finally, TGF-β expression was also measured by studying ICC images. Obtaining the images was difficult due to optimization problems that led to the day 5 pictures being unable to use. The changes in fluorescence in these showed a significant difference in TGF-β availability in

the ischemic populations in comparison to the control and inhibitor populations. These results may have been muted in the experiment due to the population not being pure. For ICC, no invasion chambers were used, just cells left in wells and exposed to DCD conditions, so the isolated functionally mesenchymal population were not used to measure TGF-β expression. This means the results were likely weaker in comparison to what the functionally selected mesenchymal population as it includes all the cells, including the non-migratory cells. All three of these techniques help indicate the strong corollary role of TGF-β in EMT. The next step was to prove causation using an inhibitor.

Inhibitor Studies

Galunisertib is a TGF-βR1 small molecule antagonist that has been shown to be effective in the prevention of EMT from occurring. It also has some inhibitory effects on TGF-βR2, albeit at a much higher concentration. Effects of inhibiting this receptor likely wouldn't cause problems interpreting the effects as TGF-βR2 activates expression of TGF-βR1 so the promiscuity would amplify the biological effect. This inhibitor was used in two separate experiments in order to observe its effects on cell migration after DCD ischemic conditions were applied. The inhibitor, when applied to the migration chambers, prevented the cell migration seen via cell count in non-inhibitor migration assay in comparison to ischemic group and the controls (fresh cholangiocytes, vehicle control, inhibitor control). Instead, there was a reversal of the rates of migration with a greater increase in the top epithelial population in comparison to the bottom mesenchymal. The inhibitor was again applied to another cholangiocyte population that underwent DCD conditions without the chamber assay. This experiment also showed a reversal of the previously seen increase in mesenchymal characteristics with ischemia. Instead of grade IV mesenchymal like cells

significantly increasing as time increased, there was a decrease in this cell group and an increase in grade I, II like that of the controls. The vehicle control of DMSO and inhibitor control showed no significant differences in cell morphology compared to the control cholangiocytes, indicating that the inhibitor and DMSO did not cause any changes themselves.

Finally, these cell populations that underwent DCD conditions without the invasion chambers also were used in ICC. TGF-β antibody binding was measured and compared to non-inhibitor ischemic fluorescence. There was a significant difference between these populations that indicates the inhibitor was working to prevent the TGF-β signaling cascade leading to further EMT progression. This was also shown by the significant difference between the inhibitor and ischemic groups in the CK-7 ICC, indicating a prevention of loss of epithelial markers. The inhibitor data indicates that TGF-β serves an important role in initiating and perpetuating the EMT signaling cascade that leads to fibrotic changes. This is key for further investigation of TGF-β being a therapeutic target for inhibition to prevent ischemic cholangiopathy in DCD livers.

Limitations and Future Studies

There were several limitations on this project due to COVID-19 restrictions and technical problems in performing experiments. The restrictions prevented lab work for almost three months and required samples to be tossed before preservation and then caused a number of delays with reopening in obtaining equipment and performing experiments. Other experiments simply would not work after repeated attempts. Limitations surrounding the study itself mainly focus on strength of the study and translatability to clinical practice. Since the study is focusing solely on cholangiocyte reaction to ischemia, there is no knowledge of the interaction with other cell populations that could influence or mediate the EMT response seen or alter TGF-β expression.

Because of this, there are more experiments to be done to build clinical significance. The strength of the study is also a limitation because of the lack of information on the proposed down-regulation of the TGF-β signaling pathway in EMT. The main points that show TGF-β plays a role in initiating EMT, and not just correlation, is the reduction of migration and decreases in TGF-β effect with inhibitor. This leaves some gaps in the knowledge to be filled.

Proposed future experiments would begin with the planned alternate TGF-β receptor inhibitor of a distinctly different structural build then Galunisertib to show that the inhibitor effects were not simply a result of the inhibitor interacting with other signaling pathways. Next would be looking at the down-stream effects, like whether SMAD4 phosphorylation is increased during DCD conditions and decreased when inhibitor is applied. Repeating the western blot and RT-PCR would likely help increase the strength of the study. Next, in preparation for clinical application, inhibitor studies to find optimal concentrations should be done followed by animal models such as rat liver syngeneic transplantation. These experiments would help overcome the limitations mentioned and enable a path toward clinical relevance to help reduce IC in DCD patients

CONCLUSION

In conclusion, the purpose of this study was to identify what role TGF-β plays in the epithelial to mesenchymal transition of primary human cholangiocyte cells following ischemic DCD conditions. RT-PCR, western blot and immunocytochemistry were used to determine correlation between EMT and TGF-β. There was a clear increase in expression of TGF-β that provoked further investigation. An inhibitor study was then done, and results showed a significant decrease in cell migration and morphology change in comparison to non-inhibitor DCD conditions. These reductions in EMT show TGF-β plays a key role in inducing EMT in human cholangiocytes. Further studies will allow clarification of the changes to the signaling pathway as well as strides toward clinical relevance. The DCD conditions and experimental model were based on identifying the underlying mechanisms of ischemic cholangiopathy in order to one day reduce its occurrence. Reducing the complications of utilizing a DCD liver will allow more donated livers to be considered viable and therefore more patients helped.

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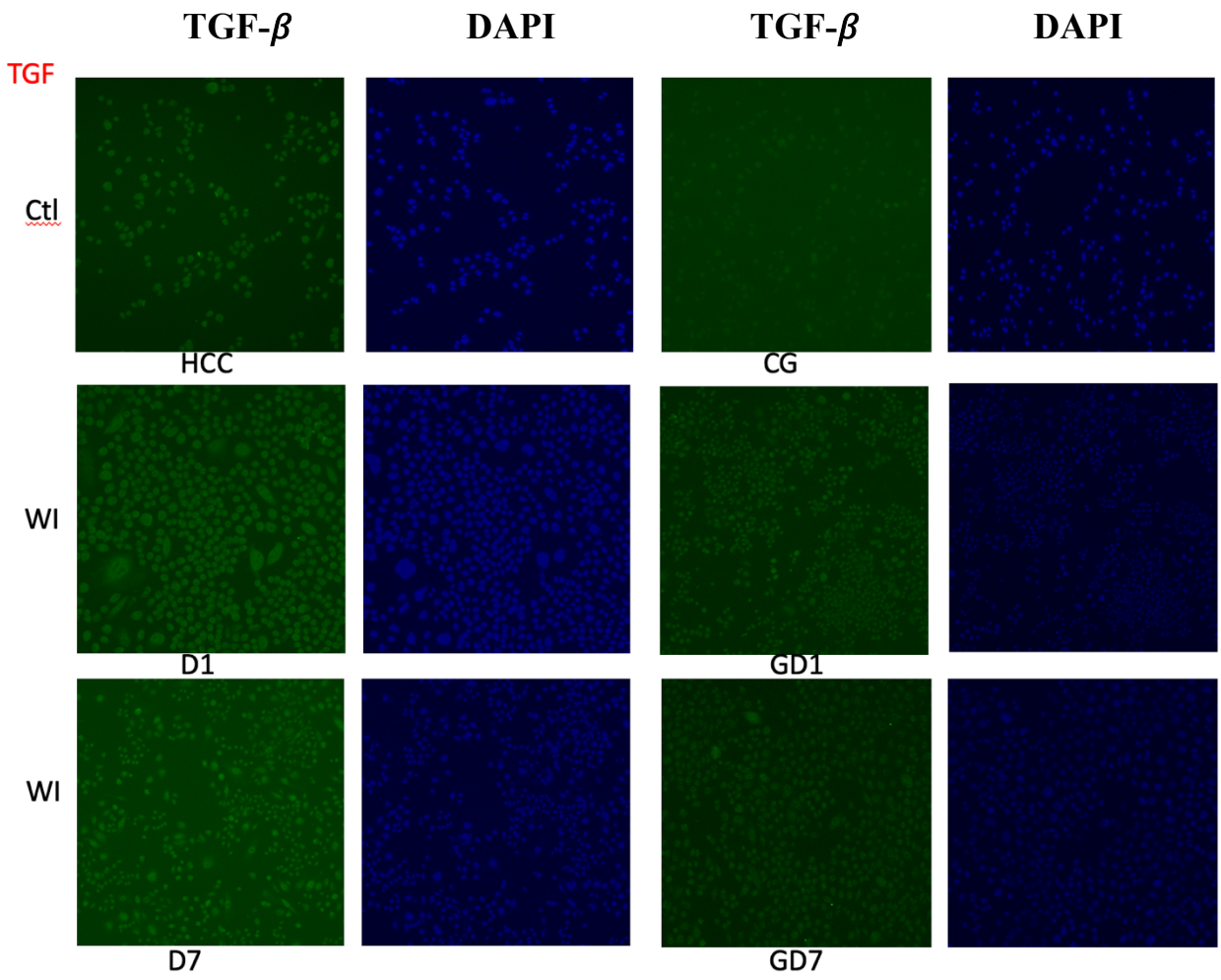
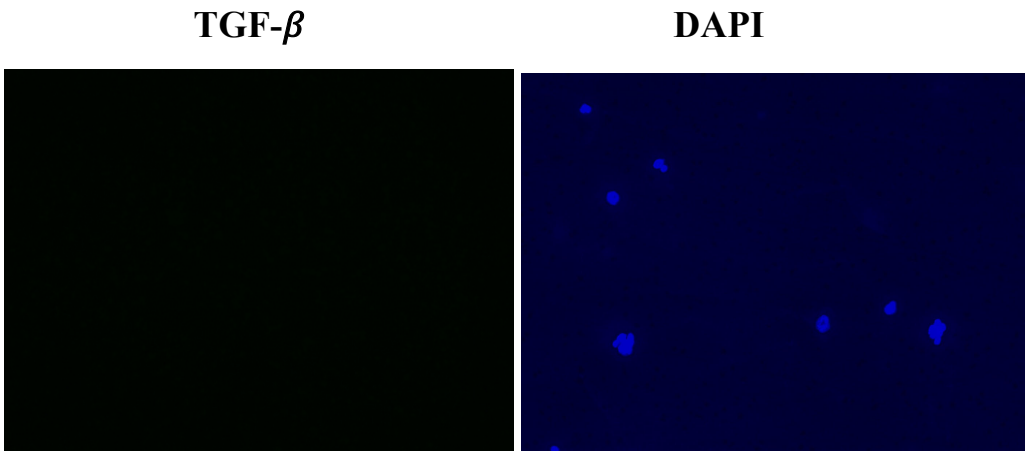
Appendix A

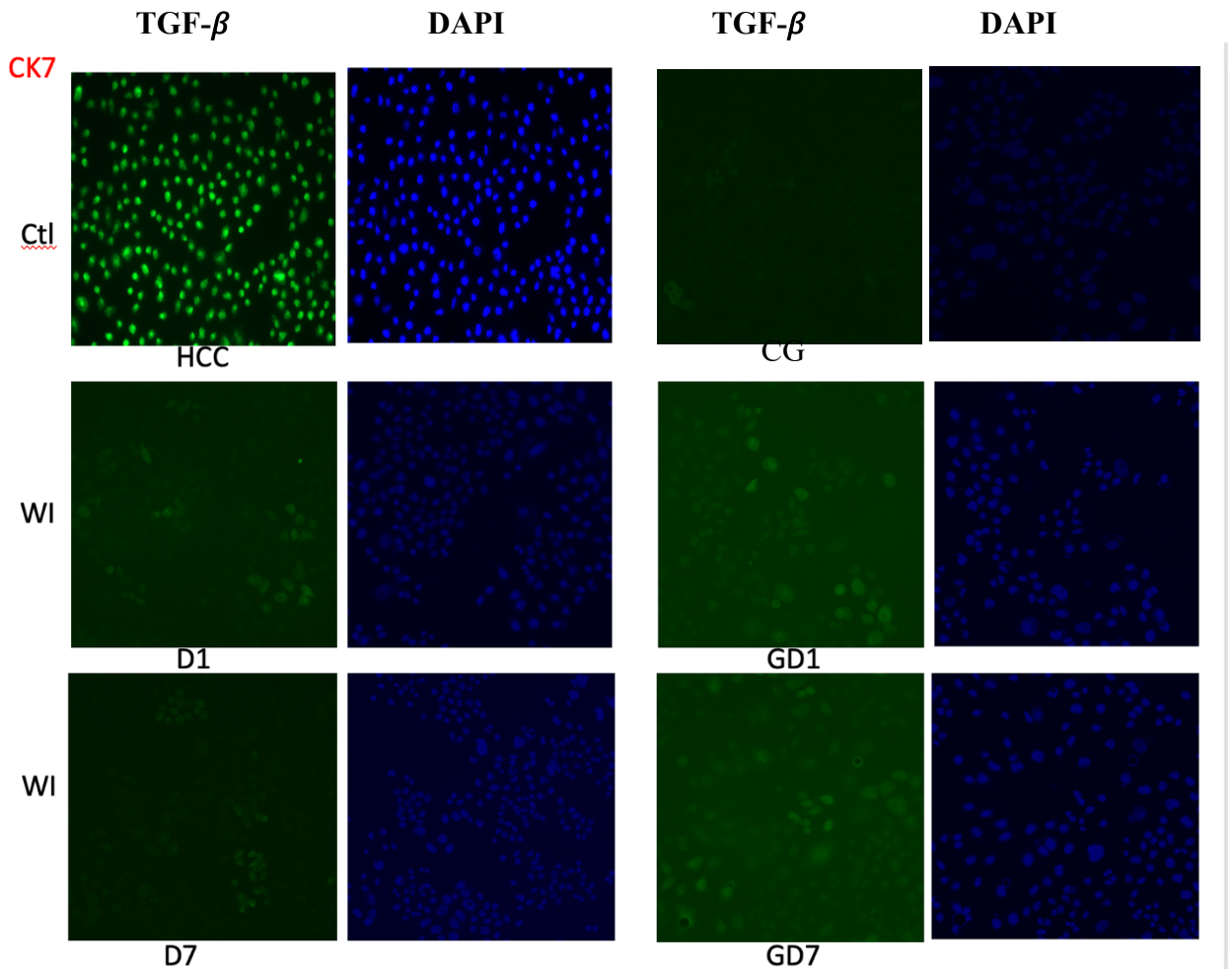
TGF-β Immunocytochemistry Fluorescence Imaging



Secondary Antibody Control



CK-7 Immunocytochemistry Flourescene Imaging



VITA

Courtney Chmielewski was born in Spotsylvania, VA in 1996 and graduated from Spotsylvania High School in 2014. She went on to attend Virginia Polytechnic Institute and State University for her undergraduate career from which she obtained a Bachelors of Science in Human Nutrition, Food and Exercise in 2018. Since then she has pursued a graduate certificate and a masters in Physiology and Biophysics. She has been admitted into the honor society Phi Kappa Phi, from which she was awarded a scholarship for excellence in her graduate coursework. She has acted as a tutor and TA for physiology this past year.