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Role of Histone Deacetylase (HDAC) in Epithelial to Mesenchymal Transition (EMT) in a Human Cholangiocyte Model of Ischemic Cholangiopathy

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A thesis submitted in partial fulfillment of the requirements for the degree of Master of Science at Virginia Commonwealth University.

by

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LIST OF ABBREVIATIONS

DBD - Donation after Brain Death

DCD - Donation after Circulatory Death

IC - Ischemic Cholangiopathy

ITBL – Ischemic-Type Biliary Lesions

EMT – Epithelial to Mesenchymal Transition

MET – Mesenchymal to Epithelial Transition

TA/IF – Chronic Allograft Tubular Atrophy/ Interstitial Fibrosis

HDAC – Histone deacetylase

HDACi – Histone deacetylase inhibitor

TSA – Trichostatin-A

RT-PCR – Reverse Transcription Polymerase Chain Reaction

ICC – Immunocytochemistry

HIF-1α – Hypoxia-inducible factor 1-alpha

ABSTRACT

Organ transplants are a vital intervention for many diseases that result in end stage organ failure. Currently, the donation pool is not meeting the demands of the transplant list. Expanding this pool to include donation after cardiac death (DCD) is highly sought-after. However, the use of DCD livers can lead to increased odds of graft failure and ischemic cholangiopathy. The loss of epithelialization and fibrosis that occurs during ischemic cholangiopathy is characteristic of these cells undergoing epithelial-to-mesenchymal transition (EMT). The biological changes the cell experiences enhance migratory capacity, invasiveness, and increased resistance to apoptosis. Our earlier studies have shown differential gene expression patterns in these cells, consistent with an EMT, after they have been exposed to conditions associated with DCD liver donation (cold and warm ischemia). In that, epigenetic modifications by HDAC can influence E-Cadherin gene expression, which is thought to be necessary for EMT. We sought to investigate the role of HDAC in cholangiocyte EMT, evoked by exposure to DCD conditions. Cell migration assays were performed with human cholangiocytes exposed to warm and cold ischemia (DCD conditions) and tested for HDAC expression with relevant gene/proteins using RT-PCR and immunocytochemistry. The effects of HDAC on EMT genotype, migratory behaviour, and cell morphometry characteristics was determined by using two structurally different, but selective HDAC-1 inhibitors, Trichostatin-A and Romidepsin. Human cholangiocytes displayed migratory behaviour following exposure to DCD conditions. Cell morphology also transitioned with a loss of epithelial and cuboidal characteristics, and a gain of mesenchymal, spindle-shaped characteristics. The use of both selective HDAC inhibitors prevented these changes in ischemic cholangiocytes and reversed changes in mesenchymal cells, changed by an existing EMT – they induced an MET. Our findings indicate that HDAC plays a major and causative role in the transition of cholangiocytes into mesenchymal cells exposed to DCD conditions. These data have implications for liver preservation in DCD donors.

INTRODUCTION

Organ Donation

Organ transplantation is the desired and beneficial for patients affected by terminal organ failures. Transplantations provide a second chance at life for patients in improved survival and quality of life.[1] Unfortunately, the number of patients needing a transplantation exceeds the availability of transplants. At the start of 2020 in the United States, approximately 25,000 candidates were listed for liver transplant. At the end of the same year, an astounding 11,772 candidates were still waiting. The scarcity of organs resulted in removal of 18% of patients from the waitlist due to pre-transplant mortality or because the patient became too sick to transplant.[2] This has increased awareness for the need to expand the donor pool and improve pre- and -post surgical outcomes for transplant patients. The rate of donation is influenced by many factors, including, funding for organ donation programs, intensive care capacity, and public awareness.[3] Some considerations for this initiative include improving organ preservation solutions and conditions at the time of transplant, decreasing the number of organs discarded, and sourcing outside the standard pool of transplants from brain dead and living donors.[4]

Organ Donor Types

There are two donation types, living donor and deceased donor donations. Deceased donations come from donors that are no longer living and are split into two categories, donation after brain death (DBD) and donation after cardiac death (DCD). The two are differentiated by presence of irreversible brain injury and state of circulatory arrest. DCD patients have severe and irreparable brain damage, and therefore do not meet the criteria for brain death.[4] The procurement process of DCD livers exposes them to warm ischemia time (WI) after circulatory arrest where there is no organ perfusion.[5] Unlike DBD donations, in which perfusion remains until the final

moment the organ is procured, DCD organs report more post-transplant complications and decreased utilization rates.[6] Though DCD liver transplants have increased in recent years, they maintain an astounding organ discard rate of 26.6% compared to DBD livers, at 7.1%.[5] Despite careful selection of organs, rates of graft survival are greater in DBD livers. DCD organs undergo two phases of ischemia, the first phase begins from the removal of ventilator and/or cardiac support and flushing the organ with cold preservation solution. The second phase takes place during implantation, before perfusion. Multiple ischemic phases greatly influence the rate of patient morbidity. Improvements during this first ischemic phase could significantly reduce organ damage due to ischemic injury.[4]

Increased DCD liver complications versus DBD livers are not significant in the Comprehensive Complication Index (CCI) until six months post-operation.[7] Ischemic-type biliary lesions (ITBL) are the most damaging cause for increased graft failures in DCD livers, by three-fold. ITBL is diagnosed via radiology and characterized as intrahepatic lesions or strictures which mainly occurs due to an ischemia-related injury.[10] Of the many causes of graft dysfuntion, the most impactful occur from acute renal failure, early allograft dysfunction, recurring hepatitis C virus (HCV), and severe biliary necrosis.[8,19] Increased graft loss rates are due to organ exposure to warm ischemia time greater than 30 minutes.[9] Hypoxia-generated reactive oxygen species, alongside the hypoxic phase during warm ischemia time initiate signaling pathways and pro-fibrotic cytokines.[11] These factors result in increased reperfusion complication for the organ. Specifically, the onset of hypoxia prior to circulatory arrest is related to the severity of hepatic reperfusion injury. Cellular injury occurs in the presence of copious amounts of oxygen that is unexpectedly available when pathways are overworked to forage oxygen-free radicals.[15] These mechanisms support the

development of biliary lesions and graft loss, as seen in a disease process termed ischemic cholangiopathy.

Ischemic Cholangiopathy

Ischemic Cholangiopathy is characterized as a set of disorders including multiple diffuse intra-hepatic strictures or non-anastomotic, affecting the graft biliary system.[13] The strictures are seen on imaging without any observable cause, including a patent hepatic artery or exclusion of a hepatic artery thrombosis, or stenosis. Symptoms are progressive in nature and generally begin months after transplantation. Patients with ischemic cholangiopathy can present non-specific symptoms or even be asymptomatic. Therefore, IC is only diagnosed when liver exams present elevated levels of serum alkaline phosphatase and gamma-glutamyl transferase.[13,14] Development of the disease may lead to cholestasis, resulting in gall stones, biliary sludge and casts, obstruction, and cholangitis.[15] Symptoms consistent with liver failure, such as jaundice and itching, may present later. The diagnosis of this condition is done via abnormal liver tests and visual confirmation of the intra-hepatic strictures in the liver by magnetic resonance imaging or endoscopic retrograde cholangiography (Figure 1).[13,16]

Figure 1. Ischemic Cholangiopathy

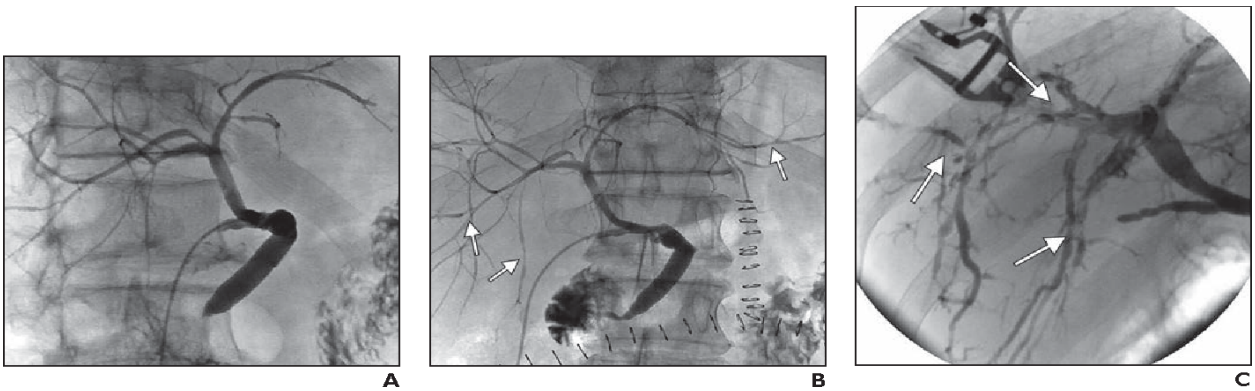

Figure 1: Ischemic Cholangiopathy seen in the cholangiography of the bile duct (including intra-hepatic) of a 59yo male; from left to right the arrows show multiple progressing stenoses after a DCD transplant.[16]

Patients diagnosed with IC experience greater readmission rates, lengths of stay, additional procedures to alleviate symptoms, and a higher rate of re-transplantation in DCD livers by three-fold.[17,18] The rates of ischemic cholangiopathy in DCD livers are between 16% and 29% in comparison to DBD livers between 3% and 17%.[14,17,18] The severity of this condition and its symptoms leads to poor quality of life, and is a major red flag regarding the use of DCD livers.

Considerable research has been put into understanding the fundamental mechanisms of IC, and ways to prevent or improve its outcomes. Ischemic cholangiopathy has three established mechanisms of action: ischemia reperfusion injury, immune response, and cytotoxic injury from bile salt toxicity.[10,20-23] These mechanisms occur simultaneously, and the resulting impact is the development of fibrotic epithelium and strictures, as seen in IC. During the anoxic period of ischemic reperfusion injury, cholangiocytes have decreased tolerance for oxygen and as a result, the epithelium is reoxygenated. The biliary epithelium is overwhelmed with toxic oxygen species formation and worsened by low levels of glutathione, a defense against these species. During this, oxygen free radicals negatively react with surrounding tissues and other macromolecules.[24] Ischemic injury, followed with increased cold ischemia time may be detrimental to cholangiocytes, the blood supply surrounding the intra-hepatic bile ducts, and the biliary epthelium.[23]

Modifications of these mechanisms may change the outcomes of IC, and even prevent its occurrence. Past studies show that a decrease in warm and cold ischemia time, as well as using thrombolytic flush on liver grafts, can significantly reduce the onset of ischemic cholangiopathy and biliary complications.[25-27] Thrombolytic flush utilizes thrombolytic tissue plasminogen activator (tPA) flush to significantly decrease the incidence of ischemic type biliary lesions.[28] Low viscosity perfusion of the hepatic artery and peribiliary capillary plexus prior to transplant has alleviated symptoms leading to IC.

Much debate has surrounded the efficacy of machine hypothermic or normothermic perfusion. Hypothermic perfusion has shown decreased injury in grafts, unlike regular cold storage conditions. Though normothermic perfusion has shown encouraging changes in IC, it requires further testing. [30-32] The previous interventions certainly decrease IC complications; however, the occurrence of graft loss and ischemic-type lesions is too frequent and warrants a deeper understanding of ischemic cholangiopathy and its management.

Epithelial to Mesenchymal Transition

Epithelial to mesenchymal transition (EMT) is a biological reversible process in which cells experience a loss of epithelium and gain fibrotic tissue. During the transition, a polarized epithelial cell, which normally interacts with the basement membrane, is subjected to biochemical changes that allow it to assume mesenchymal characteristics. Epithelial cells lose their polarity and cell-to-cell junctions lose their adhesion properties. Progress to mesenchymal characteristics include a new, spindle cell phenotype, enhanced migratory capabilities, resistance to apoptosis, and the ability to enter the extracellular matrix.[33] The fulfillment of an EMT is signaled by the degeneration of the basement membrane, allowing the cell to migrate away from its origin.[29] EMT and it's reversed process, mesenchymal-epithelial transition (MET), drive tissue morphogenesis and are controlled by several mechanisms, depending on the context. Cells undergoing an EMT or MET encounter changes in their proteins, adhesion, and gene expression (Figure 2).[34] Additionally, cells undergoing EMT cause cholangiocytes to move from their original position by transitioning to fibrotic muscle cells. This process is harmful to small intra-hepatic bile ducts and results in the disappearance of the bile duct itself. The movement and disappearance of these vital structures leads to renal failure. Therefore, the prevention and treatment of the EMT transition is necessary to avoid fibrogenesis.

Figure 2. Overview of EMT and MET transitions.

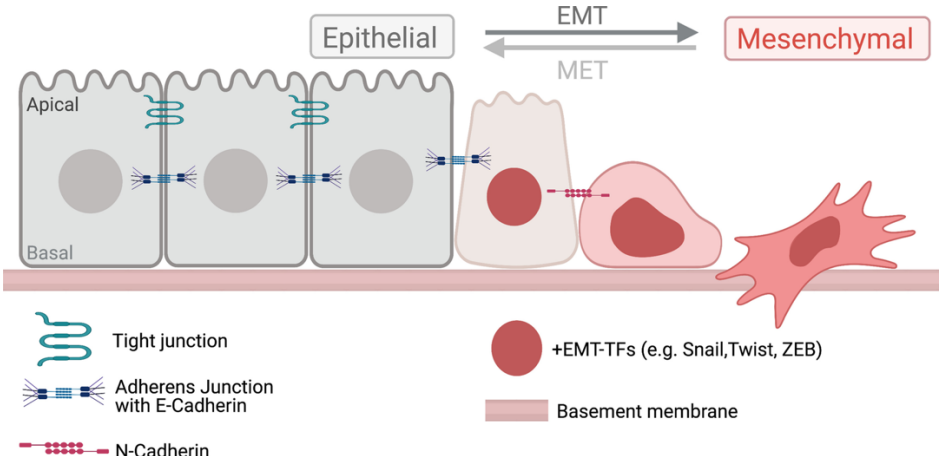

Figure 2: During EMT, cells turn on transcription factors that promote the transition. This includes disassembling cell junctions, losing polarity, and upregulating new cadherins. Cells undergo the rearrangement of their cytoskeleton, changing their phenotype. Additionally, they gain migration properties and invasive behaviors. Immunocytochemistry fluorescence images for the previously done EMT experiments.[29]

EMT may be induced in the following ways, during implantation and embryogenesis (1), during transformation of secondary epithelia to cancer cells, enabling migration and metastasis (2), and in attempts to escape mechanical stressors, injury repair, or hypoxia (3).[35] The various modes of induction make EMT a primary focus in fibrotic diseases, such as biliary atresia and primary sclerosing cholangitis.[37,38] Epithelial-to-mesenchymal transition is also seen in chronic allograft tubular atrophy/interstitial fibrosis (TA/IF), which is synonymous to IC. TA/IF occurs early after transplantation and is responsible for allograft dysfunction in the kidney and leads to myofibroblast stimulated EMT.[39,40]

The discovery of these mechanisms has led to a deeper exploration of EMT being the rudimentary cause of IC. Previously, the Dr. Mangino's lab at VCU examined morphological changes after ischemia and identified cellular marker changes in epithelial and mesenchymal cells. Their results show changes in morphology from a cuboidal to spindle shape in cells exposed to DCD ischemic conditions. Their immunocytochemistry (ICC) data further represented changes in expression of epithelial markers, CK-7 and E-Cadherin, and mesenchymal markers, SNAIL, N-

Cadherin, and Vimentin (Figure 3A, 3B).[41] Though their study was limited to cholangiocytes, they successfully identified EMT role in the development of ischemic cholangiopathy.

Additional research shows the role of epigenetic modification on gene expression, and its effect on metastasis. Inhibition of active epigenetic regulators may be effective in controlling the invasive characteristics of cells when undergoing an EMT. Current literature highlights the role of histone deacetylases (HDACs) in pancreatic metastasis, inducing EMT formation in cancer cells. HDACs enable repression of transcription by removing acetyl groups from histone tails and is involved in trypsin activation and tissue damage in acute pancreatitis.[42] The involvement of HDAC in the EMT process, along with the knowledge of identified markers warrants further investigation into its epigenetic pathways. Manipulation of said pathways may lead to improvements or reversal of an EMT.

Figure 3A. Change in Marker Expression

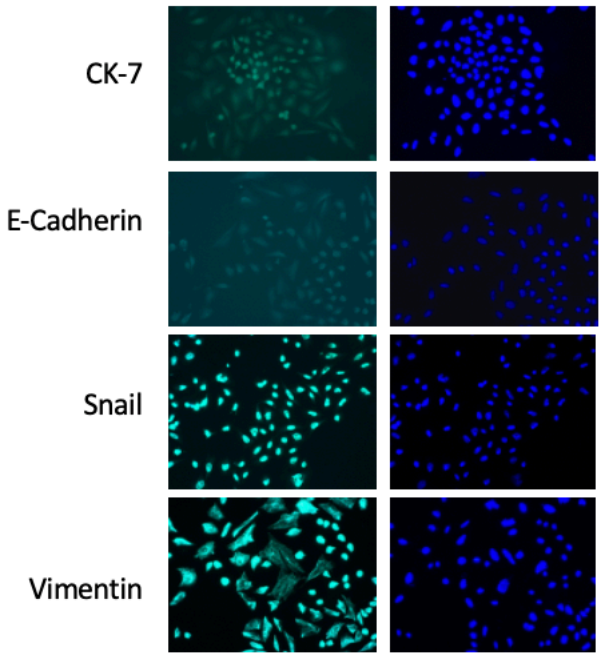

Figure 3A: Immunocytochemistry fluorescence images for the previously done EMT experiments. Results show fluorescence staining of epithelial markers (CK-7, E-Cadherin) and mesenchymal markers (Snail, Vimentin)[37]

Figure 3B. Change in Marker Expression

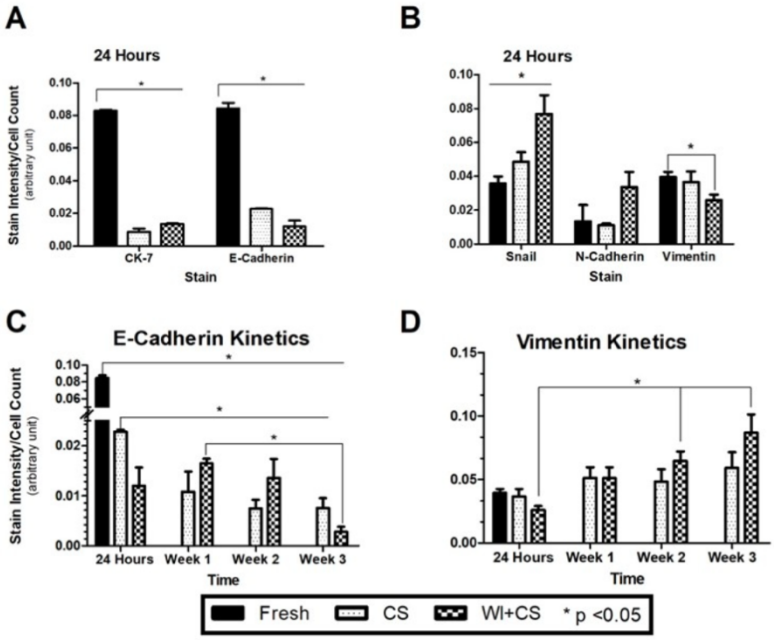

Figure 3B: Immunocytochemical expression of epithelial cell markers CK-7 and E-Cadherin (panel A) and mesenchymal cell markers SNAIL, N-Cadherin, Vimentin (panel B), comparison of fresh cholangiocytes, 24hr after cold storage (CS) and 24hr after 60min warm ischemia (WI+CS). The kinetics of expression of E-Cadherin (panel C) and Vimentin (panel D) are also shown.[37]

Histone deacetylase role in EMT

To understand the regulators of EMT and discover ways to decrease IC occurrence, the signaling pathway of EMT need to be evaluated further. HDACs are transcription repressors, which enables them to determine the acetylation status of histones. Therefore, inhibition of these HDACs may selectively alter gene transcription. Previously, Dr. Mangino's lab found that EMT occurs in a cell model of ischemic cholangiopathy. Current research shows epigenetic regulation of 2 pathways, hypoxia-induced EMT and TGF-β. HDACs have been shown to label the promoters of EMT markers, such as the epithelial mark E-Cadherin, which is the most significant in effecting cell motility in EMT.[44] A previous study showed the involvement of histone deacetylase in a hypoxia induced EMT and marked them as potential therapeutics for treating EMT (Figure 4). The studied pathway shows the repression of E-Cadherin by targeting its promoter. The pathway

defines Snail recruitment of HDAC-1/2 to mediate the repression of E-cadherin, an epithelial marker. Hypoxia inducible factors (HIF) are transcriptional regulators of angiogenesis. Specifically, HDAC1/2 has been shown to induce angiogenesis in stable HIF-1α which contributes to EMT formation via Twist, and subsequently inhibiting E-Cadherin[36]. The evaluation of the pathways shows HDAC has a role in the initiation of an EMT.

Histone deacetylase inhibitors (HDACi) have suppressed metastasis through regulation of the epithelial to mesenchymal transition in basal-like breast cancer.[46] Their efficacy lies in targeting underlying epigenetic changes which lead to malignant transformation. Currently, the use of histone deacetylase inhibitors in cholangiocytes is unknown. With the knowledge of epigenetic pathways, specific inhibitors, such as HDAC1 inhibitors may be utilized to interrupt or reverse the epithelial to mesenchymal transition.[45] Romidepsin, a selective HDAC 1 and 2 inhibitor, has proven anti-tumor treatment of cutaneious T-cell lymphoma.[47] A second HDACi, Trichostatin-A (TSA), is a selective HDAC 1 and 2 inhibitor, known for its anti-proliferative activity. It has been utilized to challenge the mesenchymal phenotype from an EMT in colon cancer.[48] TSA has also demonstrated reversal of an EMT in breast cancer by increasing the expression of epithelial marker, E-Cadherin, and decreasing the expression of Vimentin, a mesenchymal marker.[49]

Figure 4. Involvement of HDACs in the signaling pathways of cells experiencing hypoxia

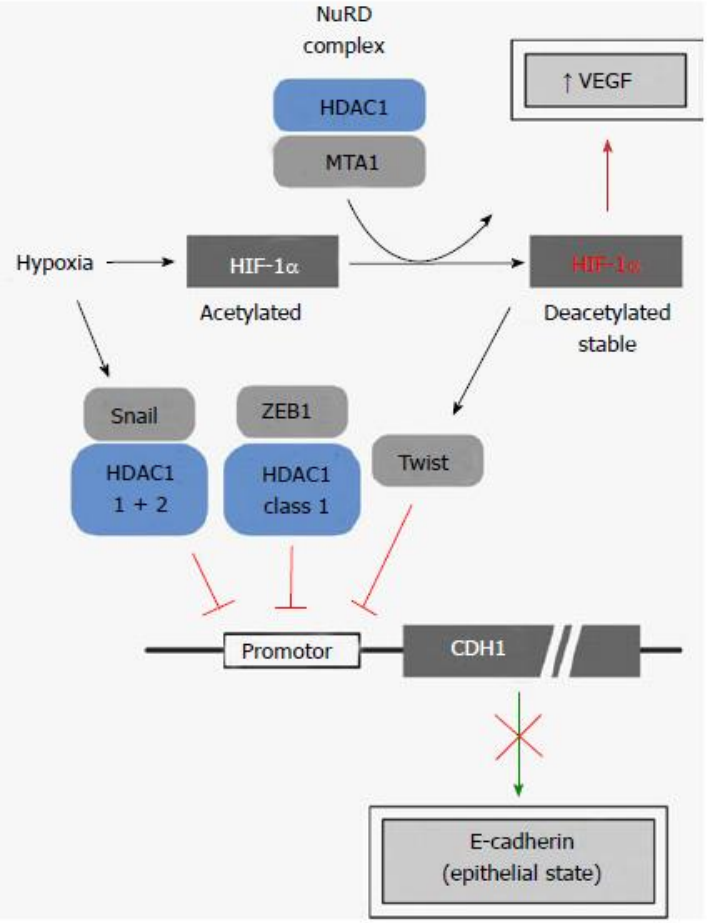

Figure 4: Snail mediates E-Cadherin repression by the recruitment of the histone deacetylase 1/2 (HDAC1/HDAC2) complex. HIF-1α is regulated and stabilized by HDAC1, which allows Twist to regulate E-Cadherin repression and contributes to EMT by a stabilized HIF-1α.[36]

Purpose of Study

The purpose of this study is to identify the role of histone deacetylases in epithelial to mesenchymal transition seen in ischemic cholangiopathy. Our earlier studies have shown EMT aiding in the loss of cholangiocytes and differential gene expression during DCD liver transplant conditions.[41] Specifically, changes in E-Cadherin were the main indicators of an EMT occurrence. To further investigate these findings, HDAC1 and HDAC2 inhibitors were used to study morphology and E-Cadherin expression in cholangiocyte cells exposed to warm and cold ischemia. With respect to previously studied pathways, influence of HDACi's on the expression of Snail, HIF-1α, and Twist were also visualized in cholangiocytes. By utilizing two structurally different HDAC-1 inhibitors, we attempted to prevent EMT from occurring. Romidepsin and Trichostatin-A were chosen due to their clinical effectiveness and selectivity for HDACs 1 and 2. Though structurally different, both inhibitors display similar methods of action during treatment.

The study replicates 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. Treatment of these cells with selective pharmacological inhibitors showed the prevention of an EMT, induction of an MET (reversal of an EMT) and decreased migratory behavior in cholangiocytes. The findings support the causative role of HDACs in the epithelial to mesenchymal transition seen in ischemic DCD conditions. All of these were measured in vitro so the possible interactions with other epigenetic pathways were 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

Human cholangiocyte cells (HCC) from primary 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). Cells were grown in an incubator with 5% CO_2 at 37°C to an 80% confluence for use. 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), antibiotic antimycotic solution (1%, Gibco), and 50% HCC media for the mesenchymal cells after ischemia storage. Cell count at various points in the experiment were conducted with a light microscope at 10X magnification, and a hemocytometer. Trypan blue was also utilized for cell counts at a 4:1 ratio.

In the basic cell model to simulate DCD conditions, cells were cultured for at least 24 hours pre-experiment to ensure sufficient attachment to the plates. Cell cultures were then placed in an airtight box (Tupperware), infused with 95% nitrogen and 5% CO_2 for 10 minutes. Next, cultures were placed in the incubator for 60 minutes to simulate the warm ischemia period between cardiac arrest and organ flushed with cold preservation solution. The airtight box is then placed in a larger airtight container and immersed in a layer of melting ice. The container is once again purged with gases and placed in a 4°C fridge for 24 hours, simulating cold storage and preservation of organs. Post-storage, cells are removed from their containers and exposed to atmospheric oxygen. Lastly, they are placed back in the incubator and cultured as normal, simulating reperfusion. Following the reperfusion, cells were collected on day 1, (for control and ischemic conditions), day 5, and

day 7. Cell populations for the basic ischemic model were fresh control cholangiocytes (CC) without ischemia, ischemic samples collected at day 7.

Cell Migration Assay

Three populations were used to observe cell migration, fresh cholangiocytes (control, HCC), EMT cells, and HDACi treated cells. To separate cell populations that undergo migration after ischemia, cells were placed within an insert, in a 12-well plate (Figure 5). Each well included a sterilized glass slide which was used to later perform immunocytochemistry. Approximately 300,000 human cholangiocyte cells, along with 0.3 mL of human cholangioctye media were placed in each insert. The surrounding well was given 0.8 mL mixture of 50% human cholangiocyte media and 50% DMEM. The cells were cultured for 24 hours to ensure attachment to the plate, and then subjected to ischemic DCD conditions, as described above.

Figure 5. Cell Migration Assay

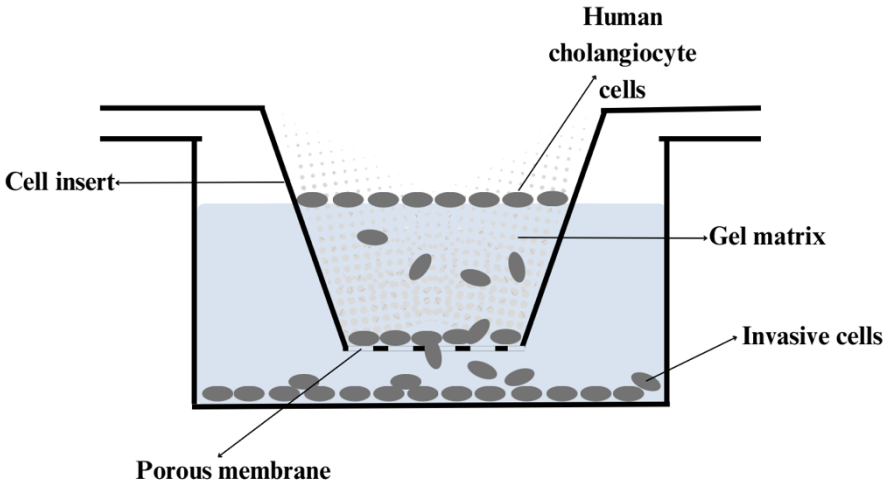

Figure 5: Cell Migration Assay to study migratory behavior after being exposed to DCD conditions over 7 days. Fresh cholangiocytes remain on top of the insert with no migration. Cholangiocytes exposed to 1hr warm ischemia and 24hr cold storage migrate through the insert onto the glass slide below.

Cells are removed from storage ischemia and once again placed in the incubator for varied timepoints to allow cell migration. Cell media was changed every three days during the collection phase. Control populations that did not undergo ischemia also were used and pulled at the same

timepoint as the day 1 culture. There were two different cell populations after the timepoints were finished: the cells that remained on top within the insert, functionally resembling epithelial cells, and the cells that migrated through the insert to the bottom of the plate, functionally resembled mesenchymal cells. To recover the top culture, a cotton swab was gently run over the surface of the insert 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, 10 uL of this mixture is mixed with 40uL of trypan blue and a cell count is performed.

After removing the top cell culture, the bottom cell population is removed by applying Trypsin for 3-5 minutes. Cell media is added to neutralize the cells and the mixture is transferred to a conical tube and centrifuged down. The mixture of trypsin and media is aspirated off and 1 mL of media added for further use. Next, 10 uL of the mixture is transferred to a tube with 40 uL of trypan blue and used for cell count. The remaining cells in media are added to new wells and cultured for further analysis via real-time polymerase chain reaction (RT-PCR/qPCR). The cell samples collected were top/cholangiocyte control (HCC), bottom/mesenchymal (HCC+DCD), and HDACi treated (HCC+DCD+RT/HCC+RT/HCC+TSA) on day 7.

HDAC Inhibitor

To determine if histone deacetylases serve a causative role, two structurally different inhibitors were used. Romidepsin (Selleck, cat. #S3020) is a potent HDAC 1 and 2 inhibitor with an IC_{50} of 36 nM and 47 nM, with a molecular weight of 540.7. Trichostatin-A (TSA) (Selleck, cat. #S1045) is another, structurally different HDAC 1 and 2 inhibitors, with an IC_{50} of 1.8 nM and a molecular weight of 302.4. A stock 2 solution for Romidepsin was made by adding stock 1

to HCC media at a 1:100 dilution, for a final concentration of 10 mM/mL in HCC media. A stock 2 solution of TSA was made by adding stock 1 to HCC media at a 1:100 dilution, for a final concentration of 2 uM/mL. Next, a 1 uM concentration of each inhibitor was applied to the cell samples. During the Romidepsin treatment, the cell populations included, fresh cholangiocyte control with HCC media, mesenchymal cells with DMEM media, and HDACi treated mesenchymal cells (50% HCC, 50% DMEM media). For TSA treatment, four cell populations were used, fresh cholangiocyte control with HCC media, vehicle control with DMSO in HCC media, mesenchymal cells with DMEM, and Romidepsin treated fresh cholangiocytes (50% HCC, 50% DMEM media), and TSA treated fresh cholangioctyes (50% HCC media, 50% DMEM media). A vehicle control was also performed using the same concentration of DMSO (add % DMSO) without inhibitor mixed in HCC media. Before cells were subjected to warm ischemia, they were allowed to incubate for 24 hours to ensure efficacy of treatment. Ischemic conditions were applied as described above. Cells then underwent cold storage ischemia and were kept for the analysis of cell morphology and immunocytochemistry (ICC). Once again, the cell populations used were, (a) fresh cholangiocyte control (HCC), (b) vehicle control (DMSO), (c) cells exposed to ischemia (HCC+DCD), (d) HDACi treated ischemic cells (HCC+DCD+RT), (e) HDACi (Romidepsin) treated fresh cholangiocytes (HCC+RT), and (f) HDACi (TSA) treated fresh cholangiocytes (HCC+TSA).

Cell Morphometry

A light microscope and trypan blue were used to observe cell morphology. Cell images were taken, and a 9-grid template was overlayed over each image. All images were analyzed randomly in to avoid bias. Cells were graded between I-IV, 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). Cells graded 'I' were most like epithelial cells, while cells graded 'IV' resembled mesenchymal cells. The numerical data was used to conduct statistical analysis of all cell populations.

Reverse Transcription Polymerase Chain Reaction (RT-PCR/qPCR)

To study gene expression, RT-PCR was performed. The samples used were collected after the cell migration assay at the specified timepoints, however technical malfunction prevented same experiment comparison. Cells were washed with PBS before beginning RNA isolation, utilizing a mixture of lysis Buffer RLT and 2-Mercaptoethanol. The homogenized lysate mixture was put through a DNA spin column. It was then put through an RNA spin column which was washed multiple times to isolate and purify the RNA. The purified sample (1 uL) were then measured in a Nanodrop One and provided concentrations (ng/uL), ratio of A260/280, and ratio of 260/230. The A260/280 ratio is an indicator of RNA purity while the A260/230 ratio measured sample purity. Each sample was done in triplicate for accuracy and the results were used to ensure the samples were sufficient to undergo RT-PCR. After this, the values were input into a 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 5 minutes at 25°C, the reverse transcriptase phase was 20 minutes at 46°C, and finally RT inactivation for 1 minute at 95°C. The samples were then held at 4°C.

Next, RT-PCR was performed with the specific primer sequences added. GAPDH, with deionized water, was added with a 5'-3'forward primer and a 3'-5' reverse primer and served as an internal control or housekeeping gene. Epithelial marker, E-Cadherin, and mesenchymal markers, HIF-1α, Snail, and Twist were analyzed with the same requisite primers. For accuracy,

each sample was done in triplicate for each gene. Once the final data was collected, the samples underwent statistical analyses.

Immunocytochemistry

The cells previously used for the migration assay were placed in wells with sterilized glass slides. Once again, the groups were, fresh cholangiocytes (HCC), cells exposed to ischemia (HCC+DCD), and HDACi treated ischemic cells (HCC+DCD+RT). 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 concentrations of antibody used for E-Cadherin, HIF-1α, and Snail were 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 data was also analyzed with t-tests to compare the ischemic group to the ischemic plus HDAC-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 RT-PCR was conducted twice due to technical errors in prepping mixtures. E-Cadherin data from the second round of PCR was not saved properly, and therefore lost. The initial cell migration assay was repeated due to failure to collect the top cell layer. Lastly, videography of cell transition was not possible due to the focal plane issues when looking through the light microscope.

RESULTS

Cell Morphology

Cell morphology was a primary outcome while studying the effects of HDAC inhibitors on cells undergoing the EMT process. Each image was divided into nine quadrants and graded. The data was calculated by grading the images based on epithelial or mesenchymal characteristics. 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). The percent of each grade out of total cells was used for the mean value. The control group, HCC, contained fresh human cholangiocyte cells that were not exposed to warm ischemia and cold storage. These cells presented strong epithelial features, such as a cuboidal appearance and small shape (Figure 6). The cells in the grade I category were very close together, and distance between the cells increased in the grades II to IV. Of note, the control group reported some migration and had a small number of cells with mesenchymal features, in the grade III and IV category. The second group, HCC+DCD, contained cells that were

exposed to warm ischemia and cold storage. This group had many cells with distinct mesenchymal characteristics, such as an elongated and spindle appearance. The HCC+DCD group also included cells within the grade III category, showing mostly mesenchymal features. The cells within this group showed distance between each other within the grade III and IV category. The last group, HCC+DCD+RT, contained cells exposed to ischemic conditions and then treated with the HDACi, Romidepsin. As these cells were first exposed to ischemia, they showed distinct mesenchymal characteristics before being treated. Post treatment, many cells were phenotypically similar to an epithelial cell type, changing their original mesenchymal phenotype. Many cells were small and distinctly cuboidal in appearance. This group also included cells that appeared mostly mesenchymal (grade III), or distinctly mesenchymal (grade IV), with the elongated, spindle-like structure. Further, the epithelial-like cells were close in proximity while mesenchymal-like cells had special distance in between.

Figure 6. Cell Morphology of Cholangiocytes

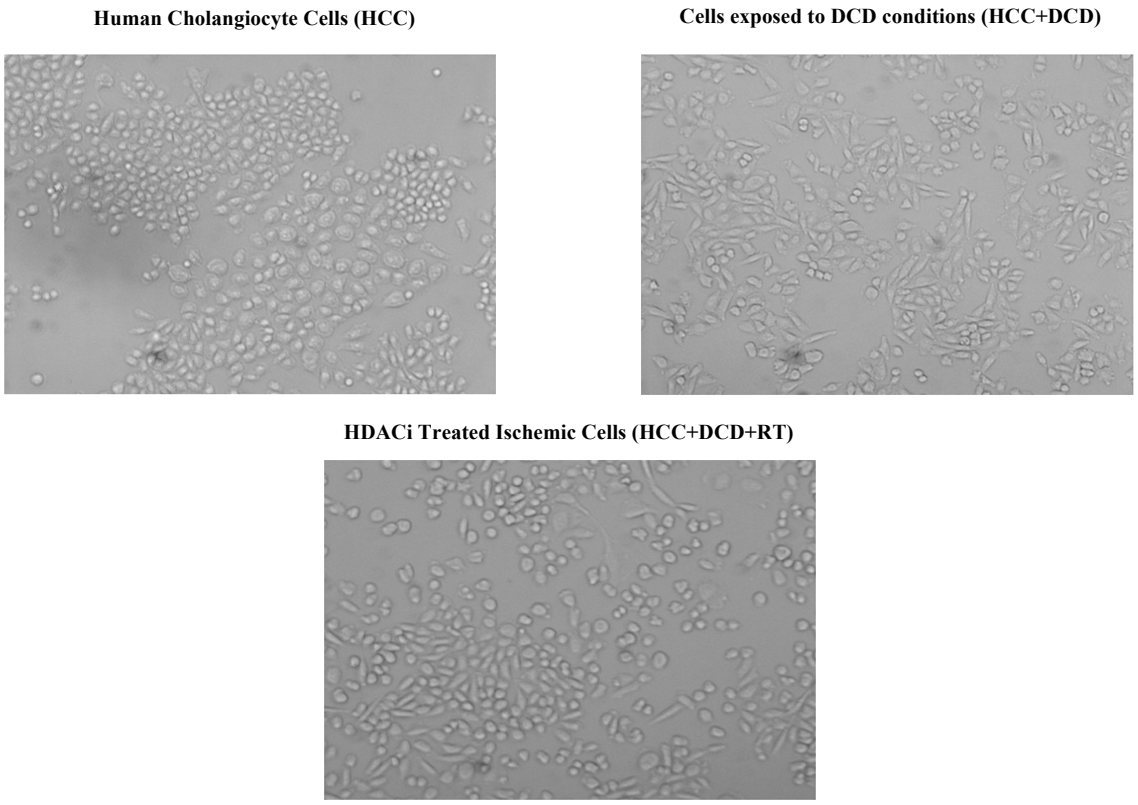

Figure 6: Cell morphology change with exposure to DCD ischemia to study the effects of an HDAC inhibitor on the EMT process. Human cholangiocyte cells displayed strong epithelial characteristics of a small, cuboidal shape. EMT cells showed strong mesenchymal characteristics such as an elongated, spindle appearance. Romidepsin treated EMT cells showed a mixture of cells, with the majority having strong epithelial characteristics.

The HCC group that did not undergo ischemic DCD conditions showed significance in the grade I epithelial category by 70% with less than 10% of cells in the mesenchymal, grade IV category (Figure 7). Conversely, the DCD group showed significance in the grade IV, mesenchymal category, by 40% and very little significant in the grade I, at 18%. In the HDACi treated group, cells were originally with strong mesenchymal features. In this group, 49% of cells were in the grade I category and are distinctly epithelial. By contrast, only 11% of cells were in the grade IV category, which have distinct mesenchymal features. Statistical analysis was performed using an ANOVA and Tukey test for significance of each experimental group within the four grading categories. Statistical significance in the data was seen between the grades I, III, and IV in the HCC and HCC+DCD groups, and in grades I and IV between the HCC+DCD and

HCC+DCD+RT groups. No significance was seen between the HCC and HCC+DCD+RT category.

Figure 7. Cell Morphology Results

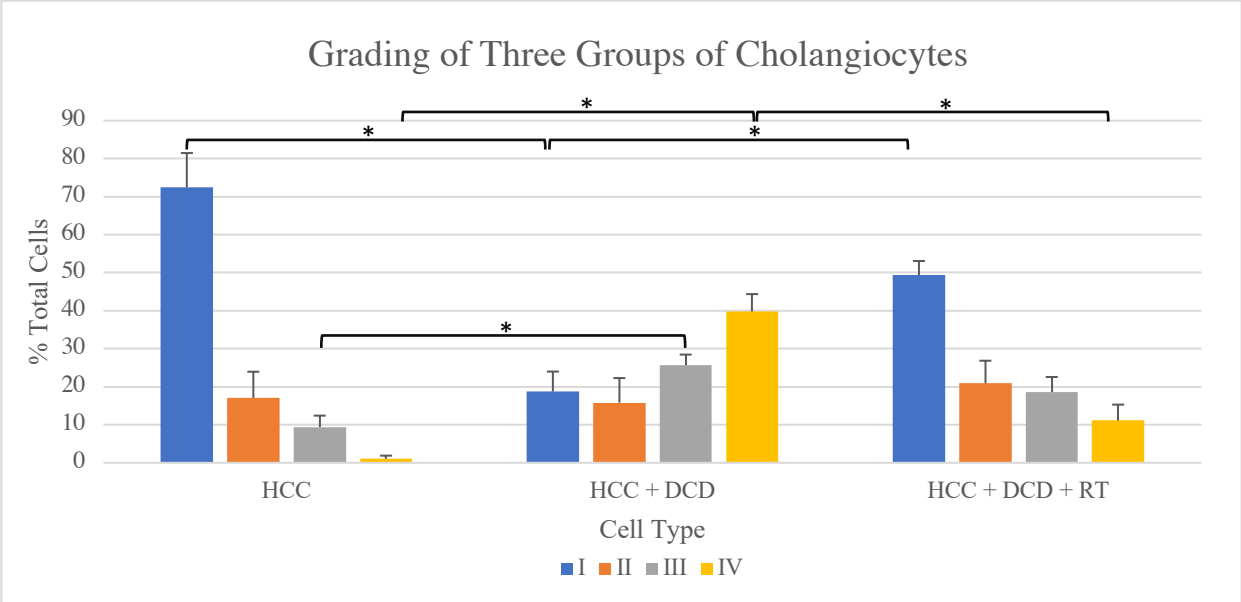

Figure 7: Cell morphology results from the grading of pictures taken with light microscopy. Data is plotted cell type against the mean percent of cells out of total cells. There are three main groups, the control group with fresh cholangiocytes (HCC), cells exposed to ischemia (HCC+DCD), and Romidepsin treated ischemic cells (HCC+DCD+RT). Significance with P value <0.05 is indicated with an asterisk. Groups HCC and HCC+DCD were significant in grades I, II, IV. Groups HCC+DCD and HCC+DCD+RT were significant in grades I, IV. I refers to cuboidal appearance, II mostly cuboidal appearance, III refers to mostly spindle appearance and IV refers to spindle appearance. N = 4

Gene Expression of HDAC 1 & 2, HIF-1α, and Twist via RT-PCR

All RT-PCR data was collected for the following cell populations, HCC (control, no ischemia), HCC+DCD (warm ischemia and cold storage), HCC+DCD+RT (ischemia followed by treatment). GAPDH served as the control, house-keeping gene. As seen via morphology, the HCC group mainly had cells with distinct epithelial features, HCC+DCD cell were mainly mesenchymal like, and HCC+DCD+RT were mainly epithelial like. First, expression levels of HDAC 1 and HDAC 2 were analyzed in populations to determine selectivity. Next, gene expression was measured for E-Cadherin (epithelial marker), Snail (mesenchymal marker), Twist (mesenchymal marker), and HIF-1α (an EMT mediator). HDAC 1 had the greatest expression in the HCC+DCD

group, followed by the HCC+DCD+RT, and lowest in the HCC control group. Conversely, HDAC 2 had greater expression in the HCC+DCD+RT group, followed by the control and HCC+DCD. Statistical analysis shows significance with HCC and HCC+DCD, as well as, HCC+DCD and HCC+DCD+RT for HDAC 1. In HDAC 2, significance is only seen with the HCC+DCD and HCC+DCD+RT cell populations (Figure 8).

Figure 8. RT-PCR Results for HDAC 1 & 2

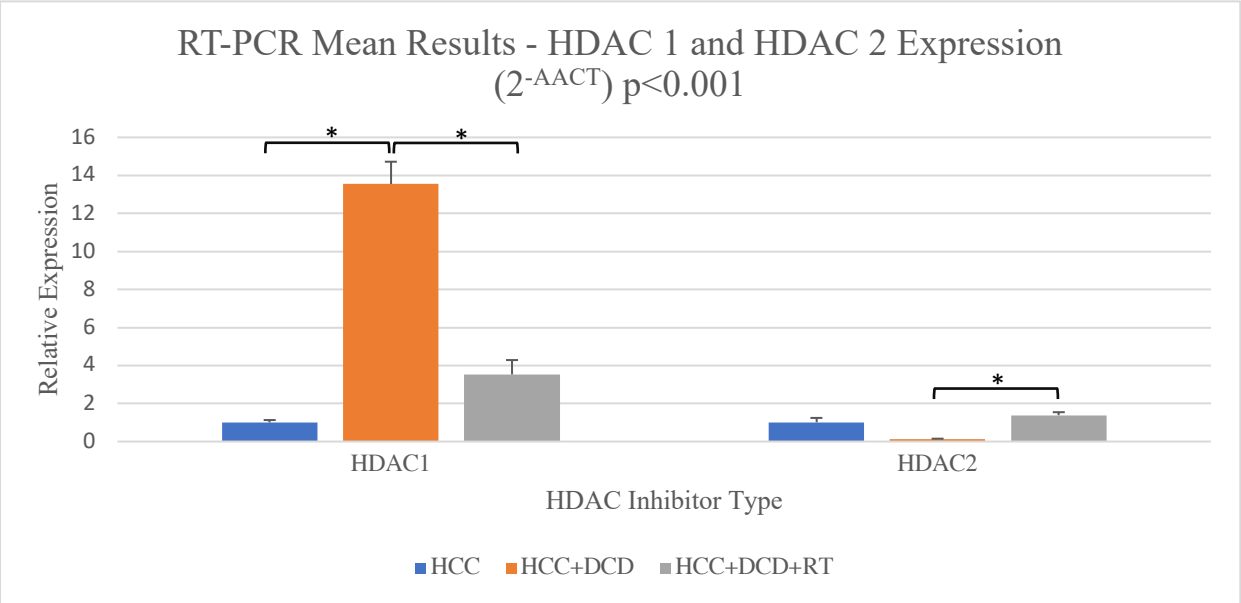

Figure 8: RT-PCR results for three cell populations on HDAC1 and HDAC2 expression. The HCC group is fresh control cholangiocytes, not exposed to DCD ischemic conditions. P value significance is set at less than 0.05 and indicated with an asterisk. Statistical significance is seen in HDAC1 for HCC, HCC+DCD; HCC+DCD, HCC+DCD+RT. In HDAC 2, statistical significance is seen for HCC+DCD, HCC+DCD+RT. N = 3.

Twist is a mesenchymal marker, and therefore should not be in high levels in epithelial cell groups. Twist gene expression was greatest in the HCC+DCD group, followed by HCC, and then lowest in HCC+DCD+RT group. Statistical significance was seen with HCC and HCC+DCD, and also with HCC+DCD and HCC+DCD+RT for Twist expression (Figure 9). HIF-1α is a strong mediator of EMT cell development. HIF-1α expression was greatest in the HCC+DCD group, and lower in the HCC+DCD+RT group. It is to note that there was extremely low expression within the HCC control (Figure 10). Statistical significance was seen within HCC and HCC+DCD, as

well as, HCC and HCC+DCD+RT. Statistical data for marker results experienced a technical complication during the back-up process for the E-Cadherin gene. However, the PCR amplification curve showed homogeneity and an increase over the baseline signal for all markers. This data supports the use of HDAC inhibitors to interrupt the pathway and effect the epithelial-to-mesenchymal transition.

Figure 9. RT-PCR Results for Twist

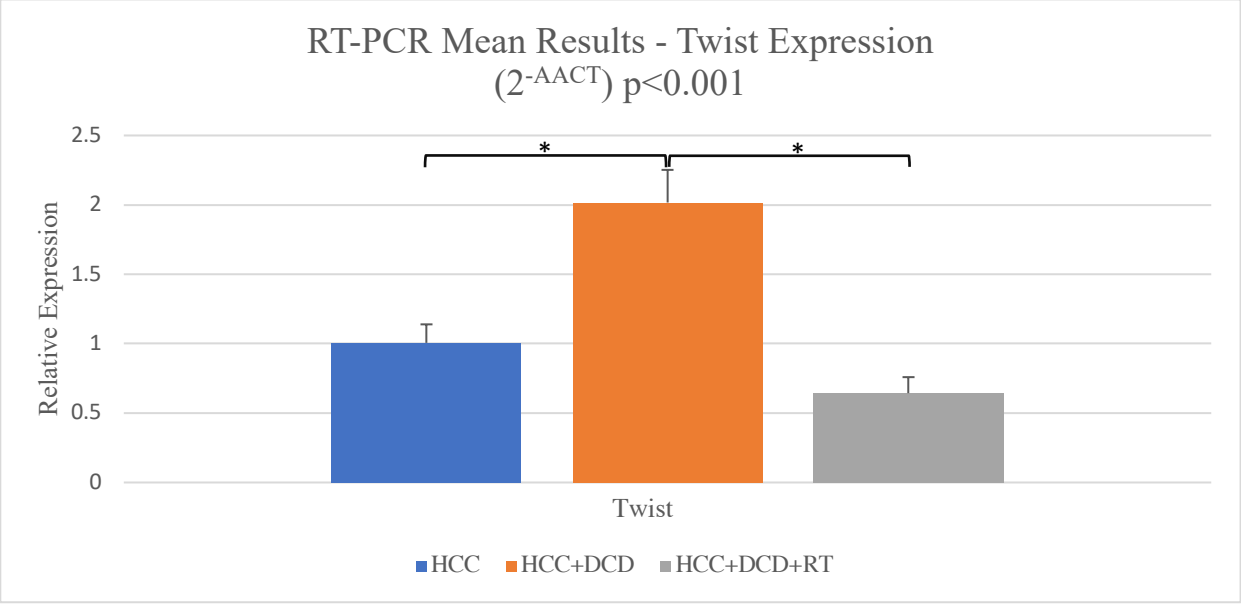

Figure 9: RT-PCR results for three cell populations on Twist expression. The HCC group is fresh control cholangiocytes, not exposed to DCD ischemic conditions. P value significance is set at less than 0.05 and indicated with an asterisk. Statistical significance is seen for Twist expression in HCC, HCC+DCD; HCC+DCD, HCC+DCD+RT. N = 3.

Figure 10. RT-PCR Results for HIF-1α

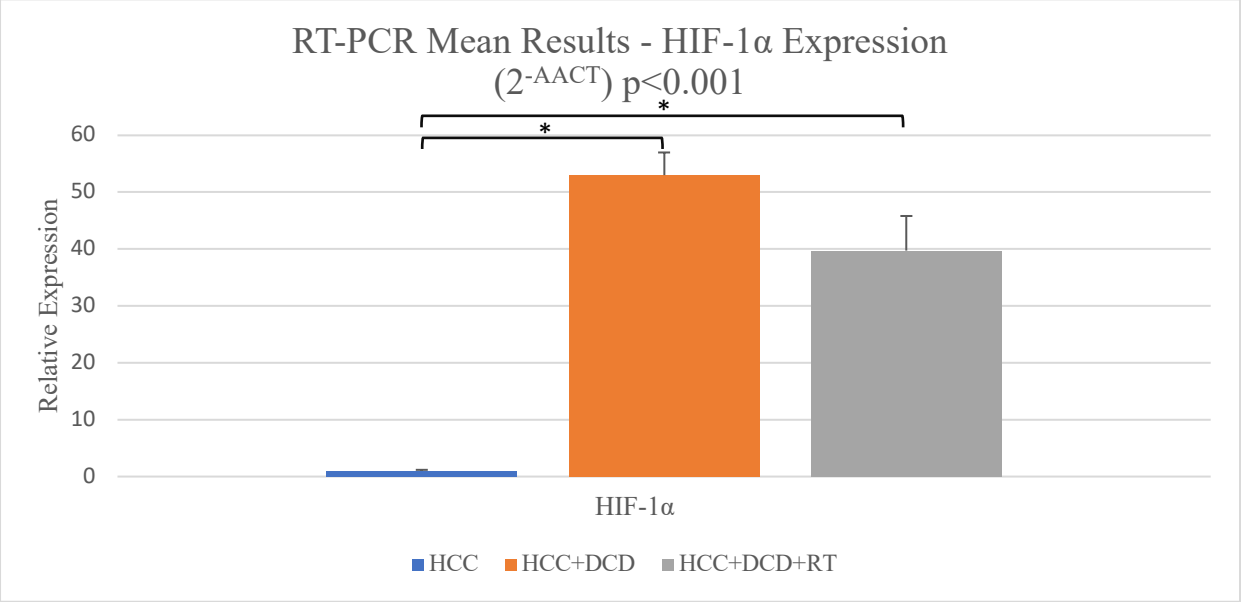

Figure 10: RT-PCR results for three cell populations on HIF-1α expression. The HCC group is fresh control cholangiocytes, not exposed to DCD ischemic conditions. P value significance is set at less than 0.05 and indicated with an asterisk. Statisical significance for HIF-1α expression is seen in HCC, HCC+DCD; HCC, HCC+DCD+RT. N = 3.

Protein Expression of E-Cadherin, Snail, HIF-1α via Immunocytochemistry

Immunocytochemistry was performed to see the differences in E-Cadherin, Snail, and HIF-1α. After undergoing DCD ischemia, the cells were measured for magnitude of staining and changes in concentration amongst HCC, HCC+DCD, and HCC+DCD+RT groups (Appendix A). Morphologically, both HCC and HCC+DCD+RT contained mostly distinct epithelial cell types, while HCC+DCD contained mostly distinct mesenchymal cell types. E-Cadherin is an epithelial marker and should therefore be prominent in epithelial cell cultures. E-Cadherin kinetics show the highest magnitude of staining in the HCC group, followed by HCC+DCD+RT, and lowest in the HCC+DCD group (Figure 11). Statistical significance is seen within all groups when compared to one another.

Figure 11. E-Cadherin ICC Kinetics Results

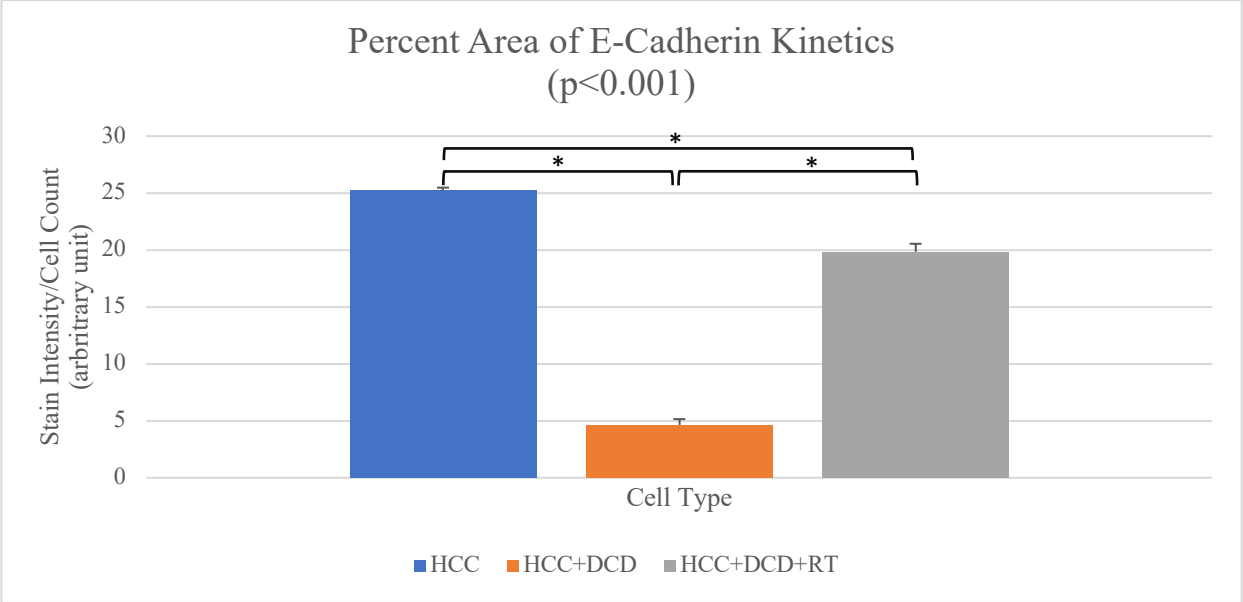

Figure 11. E-Cadherin expression, an epithelial marker, was measured in HCC, HCC+DCD, and HCC+DCD+RT cell groups. The control group included fresh cholangiocyte cells without ischemic exposure. E-Cadherin expression was greatest in the HCC group. There was a large decline in the markers expression in the ischemic group (EMT). In the cells exposed to EMT and then treated with Romidepsin, there was an increase in E-Cadherin expression (RT-EMT). Significant P value of less than 0.05 indicated by asterisk. Significance is seen between all cell groups when compared to one another.

Snail, a mesenchymal marker, should have a stronger staining within cell groups with mesenchymal features. The magnitude of staining in Snail was highest in the HCC+DCD group, which contained mostly mesenchymal cell types. Staining decreased in the HCC+DCD+RT group and was lowest in the HCC control (Figure 12). Statistical significance is seen with HCC and HCC+DCD, as well as, HCC+DCD and HCC+DCD+RT. HIF-1α is a known regulator of EMT formation, however it holds various influence within the many pathways. HIF-1α staining magnitude was greatest within the HCC+DCD cell population. It decreased in the HCC+DCD+RT category and was lowest in the HCC control group (Figure 13). No significance was seen within the groups for HIF-1α ICC. Much like the PCR data, ICC highlights the effectiveness of the HDAC1 inhibitor, Romidepsin, in reversing an implicated EMT.

Figure 12. Snail ICC Kinetics Results

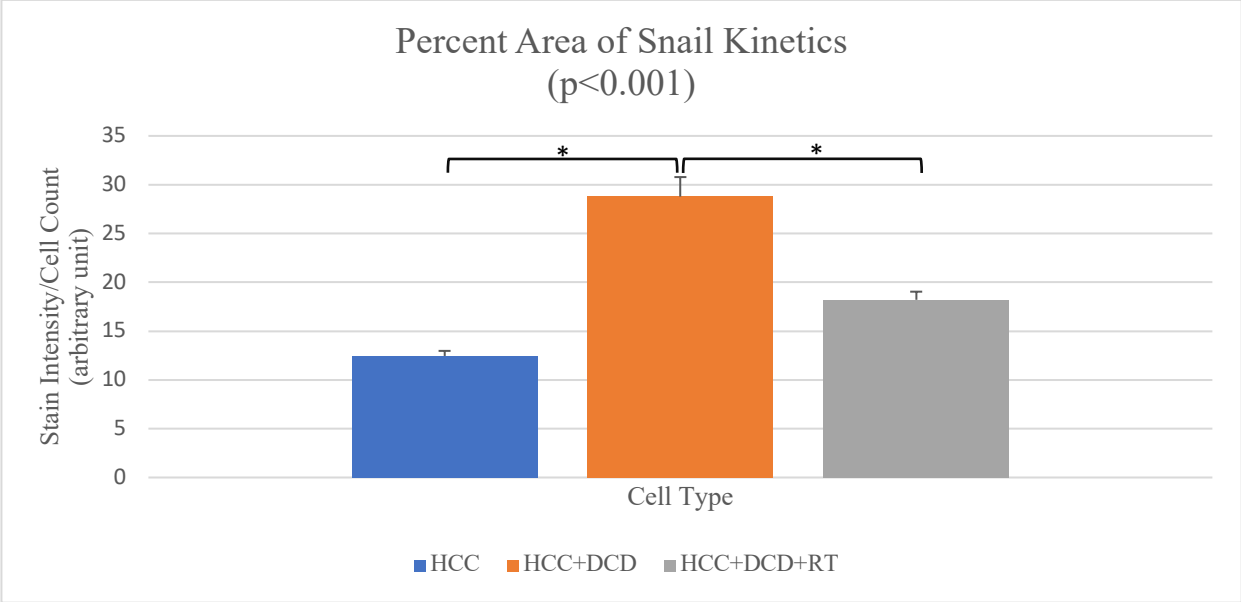

Figure 12. Snail expression, a mesenchymal marker, was measured in HCC, HCC+DCD, and HCC+DCD+RT cell groups. The control group included fresh cholangiocyte cells without ischemic exposure. Snail expression was greatest in the EMT group. There was a large decline in the markers expression in the control group (HCC). In the cells exposed to EMT and then treated with Romidepsin, there was a rise in expression, although still lower than the EMT group. Significant P value of less than 0.05 indicated by asterisk. Statistical significance was seen with HCC, HCC+DCD; HCC+DCD, HCC+DCD+RT.

Figure 13. HIF-1α ICC Kinetics Results

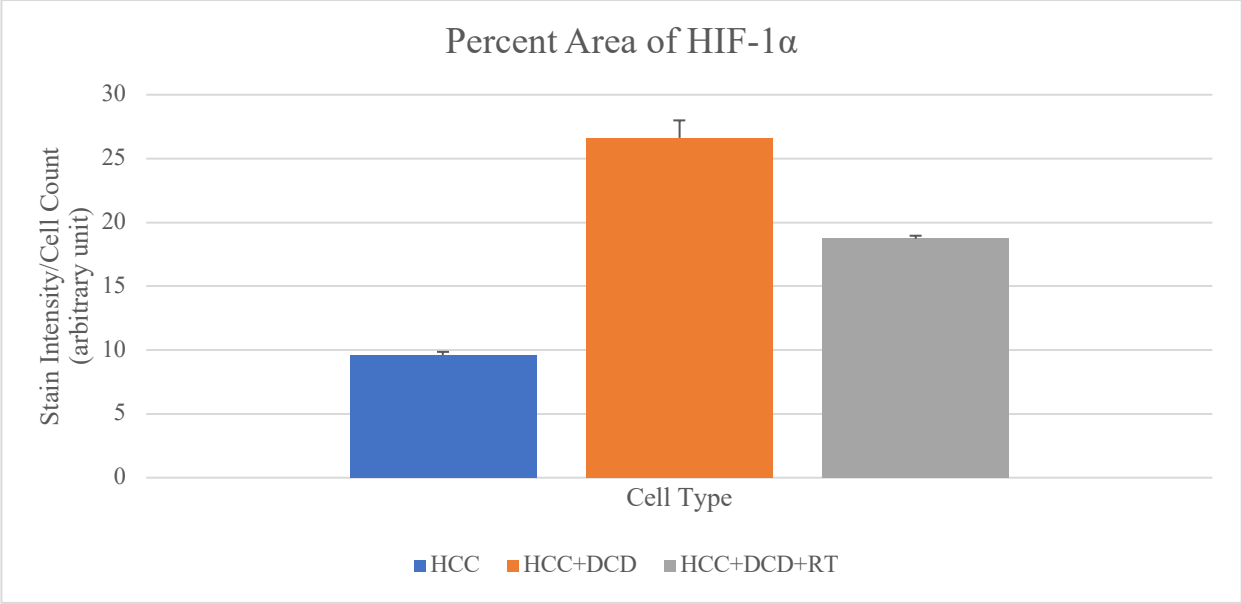

Figure 13. HIF-1α, an EMT modulator, was measured in HCC, HCC+DCD, and HCC+DCD+RT cell groups. The control group included fresh cholangiocyte cells without ischemic exposure. HIF-1α expression was greatest in the EMT group. There was a large decline in the markers expression in the control group (HCC). In the cells exposed to EMT and then treated with Romidepsin, there was a significant rise in expression, although still lower than the EMT group.

Cell Migration Assay

Cell morphology results showed the use of the HDAC inhibitor, Romidepsin, was highly effective in reversing an EMT. A second, structurally different HDAC 1 inhibitor, TSA, was used to further examine the efficacy of HDACi's. Therefore, cell migration chambers were utilized to measure migratory behavior in naïve cells, pretreated with an inhibitor before they were exposed to DCD ischemic conditions. A cell migration assay was performed with four populations, fresh human cholangiocytes cells (HCC), vehicle DMSO control (DMSO) treated HCC, Romidepsin treated fresh cholangiocytes (HCC+RT), and Trichostatin-A treated fresh cholangiocytes (HCC+TSA). The top population is functionally acting as epithelial cells and the bottom population is functionally acting as mesenchymal cells. The cells were counted on day 7, after ischemia and a hemocytometer was used to average the cells per area. The HCC control cells were collected at the same time as the day one recovery, but they were not exposed to ischemia. This group experience migration and had a large number of bottom cells, compared to the top. Next, in the DMSO vehicle group, cholangiocytes were exposed to ischemia but were not treated. This group also displayed migration with more cells being at the bottom of the chamber, versus the top. In contrast, both inhibitor groups displayed little migratory behavior, with the majority of cells remaining on top. The HCC control cells were collected at the same time as the day one recovery but without ischemia and showed some migration as well. The percent cell counts showed a decrease in bottom or mesenchymal cell population in comparison to the top or epithelial population in cells treated with an inhibitor (Figure 14). There was a decrease in epithelial to mesenchymal transition because of the inhibitor application in comparison to the vehicle DMSO cells. In the top cell population, statistical significance was seen with the following: HCC and HCC+RT; HCC and HCC+TSA; DMSO and HCC+RT; DMSO and HCC+TSA. In the bottom

cell population, statistical significance was seen with the following: HCC and HCC+RT; HCC and HCC+TSA; DMSO and HCC+RT; DMSO and HCC+TSA.

Figure 14. Effects of HDAC inhibitors on cell Counts from the Invasion Chamber starting with HCC cells 7 days after exposure to DCD conditions.

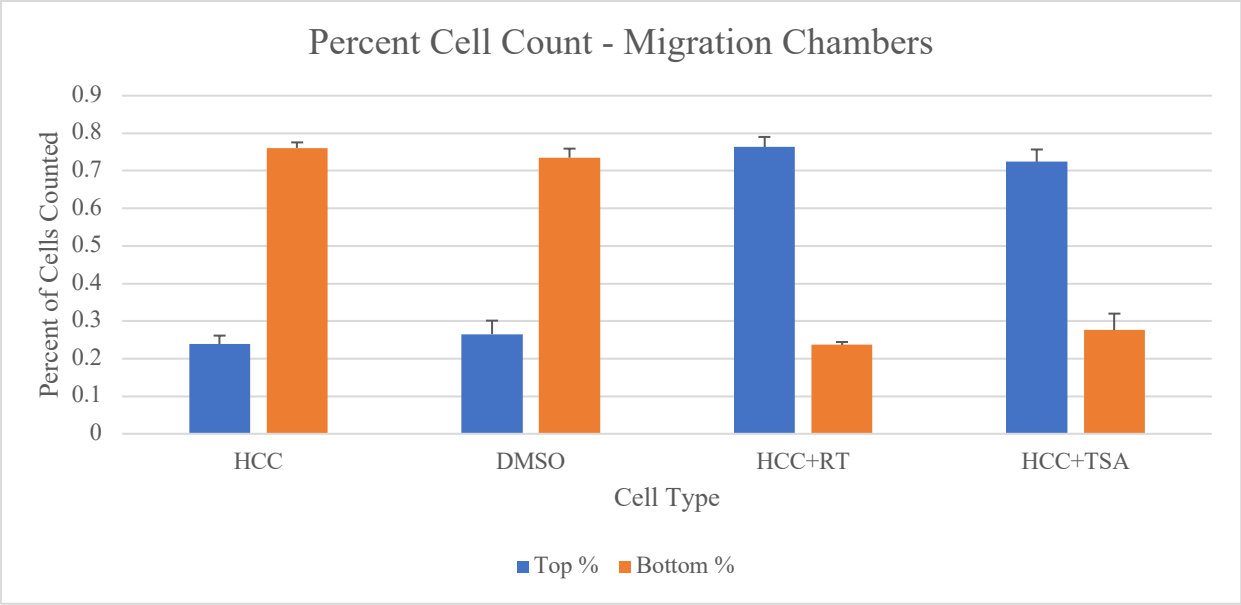

Figure 14: Percent cell counts performed for the top and bottom cell populations of the migration chambers of naïve cells treated with an HDACi. The HCC group is the control population collected 24hr after culture. DMSO cells show an increase in the bottom cell population relative to the top. Inhibitor treated groups, RT and TSA, display an interest in the top cell population relative to the bottom. Percent refers to taking the specific cell population (top/bottom) and dividing by all cells collected (top+bottom). Significant P value of less than 0.05 indicated by asterisk. Statistical significance in the top population was seen in the following groups: HCC, HCC+RT; HCC, HCC+TSA; DMSO, HCC+RT; DMSO, HCC+TSA. Significance in the bottom population was seen in the following groups: HCC, HCC+RT; HCC, HCC+TSA; DMSO, HCC+RT; DMSO, HCC+TSA. N = 2

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 increased rates of mortality. The high occurrence in DCD livers has prompted many efforts to optimize transplants in these conditions to reduce IC. However, the rates are elevated enough to warrant further investigation into the exact mechanisms to try and solve the problem with a different perspective and approach. Previously in Dr. Mangino's 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. Due to the correlation of EMT in ischemic conditions, the upcoming step was discovering if the EMT process could be prevented or interrupted by modifying epigenetic pathways.

The focus of this research is on the role histone deacetylase plays in the epithelial to mesenchymal transition that occurs in IC. Changes in hisone deacetylase expression in different cell populations were studied and there was a significant increase of HDAC1 expression when ischemic DCD conditions were applied. The trends show a strong correlation between the occurrence of an EMT in the presence of an HDACi. As such, to look more into causation, two structurally different HDAC1 inhibitors, Romidepsin and TSA, were utilized. The inhibitors decreased the migratory behavior of cells exposed to DCD conditions, as well as prevent the transition to mesenchymal appearances, and retain the epithelial marker, E-Cadherin. The results confirm that the presence of HDAC is necessary for the transition to mesenchymal cells to occur. Additional studies need to be conducted to determine the clinical significant of utilizing HDACi's, however, they permit another potential avenue to reduce, or prevent ischemic cholangiopathy in DCD liver transplants.

Phenotypic Changes

In order to better study effects of HDACs, migration chambers were used to identify migratory behavior and extrapolate the populations that transitioned. When cholangiocytes were subjected to DCD conditions, there was a discernible increase in migration which was consistant with cells having undergone an EMT. The trend represented an increased rate of cells that migrated

down through the chamber compared to the cells that remained on the top. The need for cells to migrate through the chamber symbolizes the necessary structural change that occurred for the cells to move through, such as loss of E-Cadherin protein. Of note, a small number of cells not subjected to ischemia migrated through the chamber. Although, a large increase of transitioned cells was seen with just one day of recovery time after ischemia exposure. The change in morphology that accompanied the transition was seen in previous experiments done in the lab, and the current experiment. There was an increase in spindle shaped cells, or mesenchymal cells, in cultures that underwent ischemia. Further, there were notable increases in cuboidal shaped cells, or epithelial cells within cultures that underwent ischemia with pre-treated and post-treated HDACi's. Other phenotypic changes associated with EMT and MET occurred in both groups as well. Dr. Mangino's lab has previously shown the reduction in epithelial markers, CK-7 and E-Cadherin, during EMT and an increase in mesenchymal markers, Snail and Vimentin. Therefore, only the E-Cadherin epithelial marker was used in the ICC experiment to determine if epithelial characteristics were retained. Similarly, Snail was selected, to identify if mesenchymal characteristics were kept. Cytoskeletal differences in cells during the experiment strongly indicate the formation of an EMT during ischemia, and then its subsequent reversal when treated with an HDACi. Overall, the phenotypic changes observed with DCD conditions supported and revealed the role of HDACs when manipulated with HDAC inhibitors.

Gene and Protein Expression

Following the migration chambers, cell cultures were collected to examine the expression of epithelial and mesenchymal markers. Discrimination of functional changes leading to an EMT or MET through the chambers allowed for precise selection to occur. Cells which remained on the top of the culture functionally remained epithelial, and showed an increase in E-Cadherin, but did

not show much increase in Snail or HIF-1α. The cells that migrated to the bottom functionally remained mesenchymal and therefore had an increase in Snail or HIF-1α, with little increase in E-Cadherin. This of course was not a perfect experiment, meaning there could have been cells still on the top of the chamber that were preparing to functionally change but were mid transition. This would explain slight increases in Snail or HIF-1α in the top cell samples. E-Cadherin expression was significantly increased in HDACi treated EMT cells and HDACi treated naïve cells, which is consistent with the known effect HDAC-induced acetylation has on E-cadherin promotor activity. This indicated that there is a strong correlation between ischemic induction of EMT and HDAC.

Immunocytochemistry was performed to study the changes in protein expression following DCD conditions. Visible analysis and calculation of percent cells within an area showed trends in the increase of staining intensity of E-Cadherin for Romidepsin treated DCD cells and a lack of increase in the mesenchymal population. Conversely, DCD cells displayed a greater magnitude of staining for Snail and HIF-1α in comparison to HCC and HCC+DCD+RT cells. The changes in staining intensity in the cell populations showed the significant functional changes HDACs influence in the presence of ischemia. Furthermore, data revealed the efficacy of HDAD inhibitors in reversing an EMT, causing a MET, and prevent an EMT from occurring. This could have significant implications in clinical DCD liver donation since these data show it's possible to leverage all three scenarios in the process from donation to transplantation. Specifically, potential DCD liver donors can be pretreated with HDAC inhibitors to prevent EMT or the graft, having already experienced DCD conditions, can be treated in the machine perfusion solution during organ preservation or in the recipient after transplantation. Finally, ischemic cholangiopathy that appears months after transplantation can theoretically be reversed by treating patients with early

but existing ischemic cholangiopathy. Liver transplant studies in syngeneic animals will be needed to test these translational possibilities.

All three pathways support a strong accessory role of HDAC in EMT since it seems HDAC activity is a necessary condition for EMT, likely by inhibiting E-Cadherin expression through downregulation of its promotor. To validate the HDAC inhibitor data, a structurally different HDAC inhibitor was selected for use to further support a causal role of HDAC activity in EMT.

HDAC Inhibitor Studies

Romidepsin is a class I and class 2 HDAC inhibitor that has shown clinical relevance in preventing EMT from occurring in cancer cells, specifically T-cell lymphoma. Consistent with this study, previous studies did not display drug toxicity when examining its effects on EMT and HDAC. Romidepsin was used in two separate experiments to observe and validate its effect on cell migration and morphology. During the first experiment, the drug was tested with cells that already experienced an EMT. During cell grading, the HCC+DCD+RT group showed an increase in grade I and II, similar to the HCC control. The data showed that Romidepsin was effective in reversing an EMT in cells and allowed cells to revert, causing an MET. This was also shown by the significant difference between the inhibitor and ischemic groups in the E-Cadherin ICC, indicating a prevention of loss of epithelial markers. The following experiment, done in conjunction with a second HDACi, examined cells pre-treated with the HDACi before exposure to DCD conditions. In the second experiment, Romidepsin showed inhibition of migratory behavior supporting its effectiveness in preventing an EMT. Cell count showed a reduction in cell migration to the bottom of the chamber after experiencing ischemia in comparison to the vehicle DMSO control. Instead, there was a large number of cells that remained on the top of the chamber, consistent with epithelial cell characteristics. Through visual analysis of morphology, many cells

in the Romidepsin group maintained the small, cuboidal appearance of epithelial cells. Conversely, cells in the DMSO control group consistently appeared elongated and spindle-like, like mesenchymal cells. The HCC control group showed cell morphology largely consistent with the epithelial phenotype.

Trichostatin-A (TSA) is another class I and II HDACi which is clinically remarkable due to its anti-proliferative activity. It has demonstrated success in reversing an EMT in tumor and non-tumor cells. TSA was used in the second experiment to show validity for the utilization of HDACi's in preventing an EMT. Drug potency was not toxic to any cell population. Similar to Romidepsin, naïve cells were pre-treated with TSA and then exposed to DCD conditions. Again, cell count data showed a decline in cell navigation, maintaining a large number of cells in the top level of the chamber in comparison to the vehicle DMSO control. Like the HCC control group, many TSA treated cells maintained their small, cuboidal shape, resembling epithelial cells.

Between both inhibitors, significant differences were seen in populations treated with an inhibitor versus non-treated groups (EMT and DMSO). The inhibitor data indicates that HDAC serves an important role the EMT signaling cascade that leads to fibrotic changes. This is key for further investigation of histone deacetylases being a therapeutic target for inhibition to prevent ischemic cholangiopathy in DCD livers.

Limitations and Future Studies

There were many limitations on this project due to technical problems in performing experiments. Limitations surround the study 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. Furthermore, there is only knowledge of tumor cell inhibition with

HDAC inhibitors, leaving open a large population of cell types that have yet to be studied. As such, more experiments need to be conducted to build clinical significance. The two HDACi's used, Romidepsin and TSA, were studied against specific proteins and their pathways. Therefore, more investigation needs to take place to understand the downregulation of the histone deacetylase signaling pathway in the progression of EMT. The main points that show the role of histone deacetylases in the initiation oof EMT are the reduction of migration and decrease in HDAC effect with an inhibitor. This leaves some gaps in the knowledge to be filled.

Proposed future experiments would begin with further understanding the role of HDACs within the HIF-1α pathway, such as the roles of NuRD and VEGF. Another direction includes utilizing the current HDAC inhibitors, Romidepsin and TSA, with signaling pathways of TGF-β, a component previously studied in the lab. Another favorable direction would be to analyze structurally different than the ones used, to show inhibitor effects were not simply a result of the inhibitor interacting with other signaling pathways. To increase the strength of the current study, utilization of western blots and repeating RT-PCR would help support the claim of HDAC involvement. 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 the role histone deacetylases played in the epithelial-to-mesenchymal transition in primary human cholangiocyte cells following exposure to DCD conditions. Cell morphometry, RT-PCR, and immunocytochemistry were utilized to determine correlation between HDACs and EMT. There was a notable increase in

HDAC 1 expression in EMT cells, which granted further investigation. Two inhibitor studies were done in which both sets of results showed a significant decrease in cell migration and morphology change when compared to non-inhibitor DCD conditions. The reduction and prevention of EMT show that HDACs hold a vital role in inducing EMT in human cholangiocytes. Further studies would provide clarification of the changes to the signaling pathways, as well as stride towards clinical relevance. The DCD conditions and the experimental model were based on identifying the underlying mechanisms of ischemic cholangiopathy to one day, reduce or prevent its occurrence. Reducing the complications of utilizing a DCD liver will increase the number of donated livers that will be considered viable. This would help reduce the amount of patients on the waitlist and improve post-operative conditions of livers that could potentially undergo an EMT.

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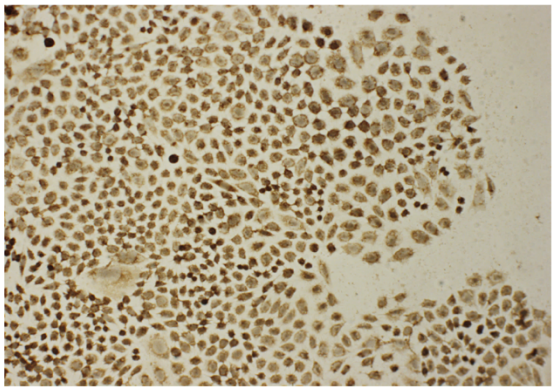
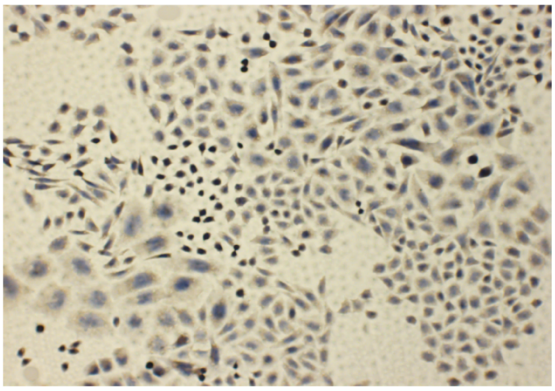
Appendix A

Expression of E-Cadherin Immunocytochemistry Staining

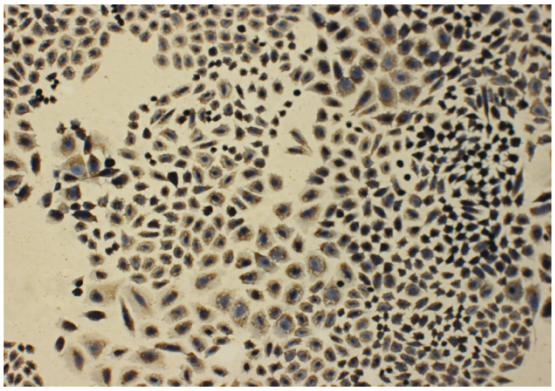
Human Cholangiocyte Cells (HCC)



Cells exposed to DCD conditions (HCC+DCD)

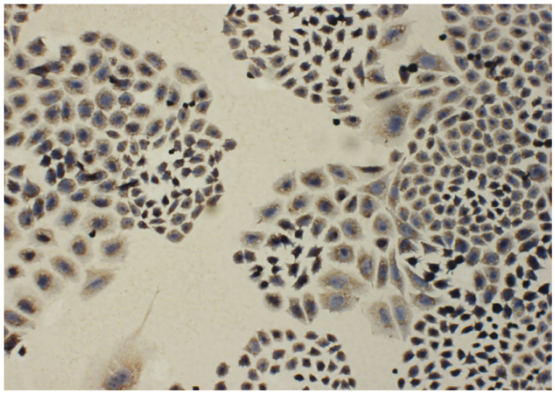


HDACi Treated Ischemic Cells (HCC+DCD+RT)

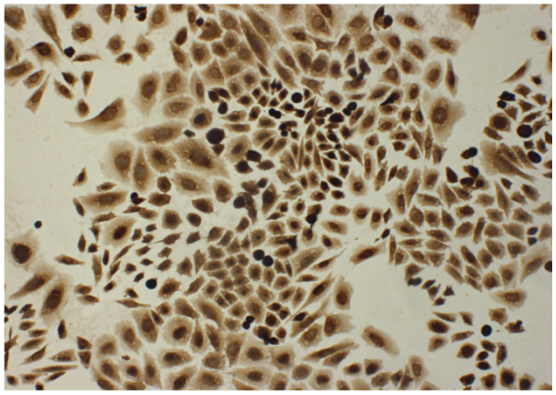


Expression of Snail Immunocytochemistry Staining

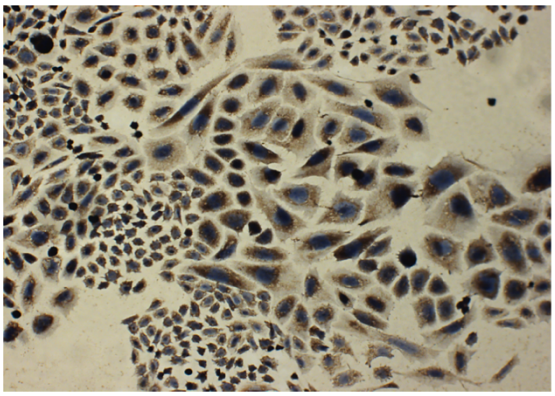
Human Cholangiocyte Cells (HCC)



Cells exposed to DCD conditions (HCC+DCD)

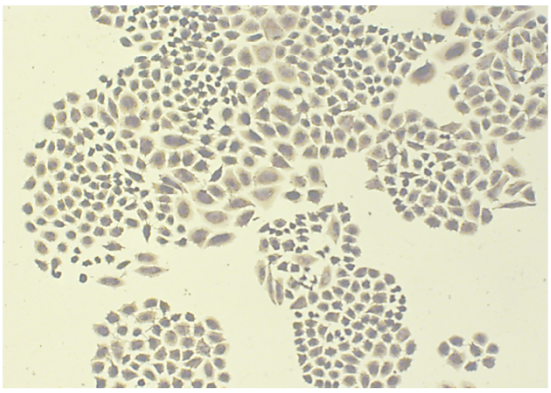


HDACi Treated Ischemic Cells (HCC+DCD+RT)

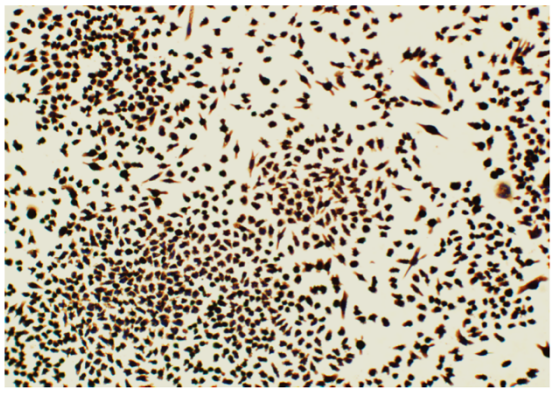


Expression of HIF-1α Immunocytochemistry Staining

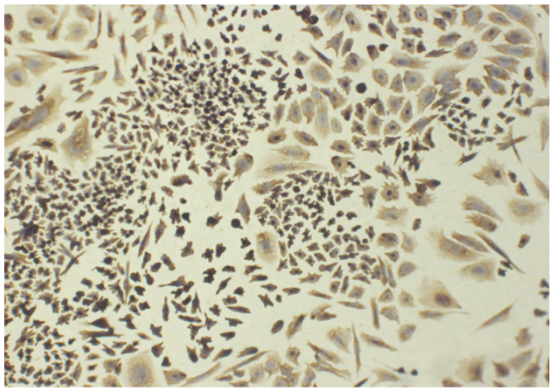
Human Cholangiocyte Cells (HCC)



Cells exposed to DCD conditions (HCC+DCD)



HDACi Treated Ischemic Cells (HCC+DCD+RT)



VITA

Priyanshi Parikh was born in Indore, India in 1998 and graduated from York High School in 2015. She went on to attend Virginia Commonwealth for her undergraduate career from which she obtained a Bachelors of Science in Forensic Biology in 2018. Since then, she has pursued a graduate certificate and a masters in Physiology and Biophysics. Along with her academia, she worked full-time and served as an intern for the Department of Surgery at VCU. She is the recipient of the HPSP scholarship through the United States Air Force and looks forward to attending medical school. She hopes to further examine her interests in research within the Air Force.