TEAM MINIGUT: DISRUPTION OF E-CADHERIN PROMOTES INTESTINAL STEM CELL PROLIFERATION IN COLONOIDS

Abstract

Reconstitution of the wounded epithelium is integral to achieve the full healing of the gut mucosa in treating Inflammatory Bowel Disease (IBD). The ability of intestinal stem cells (ISCs) to indefinitely self-renew while generating new functional epithelia makes them a potential therapeutic tool for IBD. Transmembrane protein E-cadherin, a calcium dependent cell-to-cell adhesion protein at adherens junctions, also regulates the Wnt signaling pathway. The canonical Wnt (β-catenin dependent) pathway is vital for the ISC homeostasis and regeneration. However, the role of E-cadherin in ISCs is an important yet notably understudied phenomenon. Disruption of E-cadherin increases unbound cytosolic β-catenin levels, which go to the nucleus and increase transcription of Wnt target genes. We hypothesize that disrupted E-cadherin will increase proliferation of ISCs. In our experiments, we disrupt E-cadherin with different concentrations of EGTA, a calcium chelator, and see the effect it has on colonoid growth and development. Our experiments showed that with EGTA there was greater proliferation; 1 mM EGTA experimental groups had larger colonoids than vehicle control colonoids on day 6 after seeding. This indicates that EGTA treatment may induce proliferation of the organoid with E-cadherin disruption. For future study, we will check and confirm the disruption of E-cadherin/β-catenin complex and Wnt target genes by real-time PCR and immunofluorescence studies. Ultimately our study will open novel therapeutic applications for patients living with IBD and other clinic inflammatory gut disorders.

DISRUPTION OF E-CADHERIN PROMOTES INTESTINAL STEM CELL PROLIFERATION IN COLONOIDS

Team MINIGUT

Aaliyah Arpon, Michaela Bell, Katie Brown, Alisa Forsberg, Ananya Krishnan, Ryan Margolis, Laura Marhefka, Jerry Yang, Isha Yardi

Team Mentor: Dr. Younggeon Jin

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Committee:

Dr. Andrew Schiffmacher, *Assistant Professor, College of Agriculture & Natural Sciences, UMD*

Dr. Edith Mayorga Lozano, *Post-Doctoral Associate, College of Agriculture & Natural Sciences, UMD*

Ms. Jain Kim, *Graduate Student, UMD*

Dr. Jean-Pierre Raufman, *Professor of Medicine, UMD School of Medicine*

Dr. Aditi Banerjee, *Assistant Professor of Pediatrics, UMD School of Medicine*

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Chapter 1: Introduction

Overview of the Research Problem

Inflammatory Bowel Disease (IBD) is an umbrella of autoimmune diseases characterized by chronic mucosal inflammation of the gastrointestinal (GI) tract. The GI tract is a complex aggregate of organs that coordinate various autonomous functions as part of the human digestive system. Ulcerative Colitis (UC) and Crohn's Disease (CD) are the two most common classifications of IBD. UC occurs primarily in the colon and rectum and results in continuous inflammation of the innermost lining of the large intestine (Centers for Disease Control and Prevention, 2022). While UC is typically limited to the large intestine, Crohn's Disease is characterized by patchy inflammation across the entire GI tract. The onset of IBD typically occurs in young adults, with peak diagnosis occurring between the ages of 15 and 40, however, pediatric and geriatric cases of IBD are also common (Loftus, 2011). Both UC and Crohn's disease are characterized by symptoms such as chronic abdominal pain, gastrointestinal bleeding, fatigue, diarrhea, and general gastrointestinal discomfort (Chron's and Colitis Canada, 2019).

The burden of IBD is both significant and rapidly increasing. In 2017, an estimated 6.8 million individuals were living with IBD globally (Silangcruz et al., 2021), with the age-standardized prevalence rate increasing dramatically by 4.8 per 100,000 population from 1990 to 2017 (Alatab et al., 2019). There are a number of notable demographic disparities in both the diagnosis and management of IBD. Among developing nations, India currently has the highest reported incidence rate for both general IBD and UC, with current trends suggesting that India will have the highest number of absolute IBD cases. The incidence of IBD has increased rapidly in newly industrialized countries over the past 10 years (Li et al., 2022), but recent

evidence has shown a startling increase in IBD incidence within developed nations as well (Ng et al., 2017). Currently, there is a limited body of research on racial and socioeconomic disparities in IBD incidence and prognosis. A 2019 study examining an inception cohort in the United States from 1970-2010 found that the adjusted annual incidence rate of IBD was significantly higher for Hispanic individuals compared to their non-Hispanic counterparts (Aniwan et al., 2019). Another 2014 cross-sectional study found that Black patients with Crohn's Disease had a higher incidence of Crohn's associated surgical procedures than their white counterparts (Sofia et al., 2014). Similar trends have also been observed in the relationship between socioeconomic status and IBD prognosis. A 2017 literature review on the association between socioeconomic status and IBD course found that patients with low socioeconomic status were more likely to experience higher rates of IBD-associated hospitalization and mortality (Wardle et al., 2017). While these studies and others suggest that race and socioeconomic status may be predictors for IBD incidence and prognosis, additional research is needed to support or refute these observations.

Mortality associated with IBD varies across disease manifestations. A 2022 study on temporal trends in IBD-associated mortality from 2004-2015 found that 25-30% of Crohn's Disease and 18% of UC patients died from IBD-related causes (Fakih et al., 2022). Research has also shown significant associations between IBD and comorbid disease pathogenesis. Most notably, individuals with UC have been shown to develop comorbid colorectal cancer at higher rates than the general population. A previous meta-analysis found that colorectal cancer incidence appreciated exponentially the longer an individual was living with UC, with cumulative incidence probabilities reaching 18% by 30 years of disease (Eaden et al., 2001). While IBD is broadly characterized as a disease of the GI tract, IBD has also been associated

with a number of extraintestinal manifestations. IBD patients commonly experience comorbid conditions in several other organ systems, with a previous population-based study reporting that 25-40% of IBD patients experience some extraintestinal manifestation of IBD (Bernstein et al., 2001). Common extraintestinal manifestations of IBD include other autoimmune conditions such as arthritis and psoriasis.

Current Treatment Landscape

Currently, there is no cure for IBD. Treatment involves therapeutic management and symptom control, with the goal of reducing inflammation and other symptomatic manifestations of IBD. Inflammatory cycles are commonly managed using corticosteroids (Barrett et al., 2018), a class of non-selective anti-inflammatory drugs that help to suppress immune system activity during disease flares (Waljee et al., 2016). While corticosteroids are commonly used in autoimmune disease management, they can only be used for short-term symptom control and are not an effective longitudinal treatment. Prolonged steroid use has also been associated with other serious physiological complications such as growth suppression, hyperlipidemia, and aseptic joint necrosis, among others (Buchman et al., 2001).

Immunomodulators and biological agents have become increasingly popular tools for IBD management in lieu of corticosteroids. These therapies have shown tremendous promise in improving IBD outcomes without the negative side-effects associated with long-term corticosteroid use. Immunomodulators such as azathioprine and 6-mercaptopurine have been shown to both effectively improve disease outcomes for active Crohn's disease and maintain remission (Pearson et al., 1995). Anti-TNF therapies are also popular biological agents which suppress the immune system's response to tumor necrosis factor, a key inflammatory agent

associated with mucosal inflammation in autoimmune diseases such as IBD. Several randomized control trials found Anti-TNFs to be effective agents in facilitating mucosal healing and clinical remission (Rutgeerts et al., 2005). Other biological agents such as anti-adhesion therapy and JAK inhibitors have also been effective therapeutic targets for IBD treatments.

While immunomodulators and biological agents are incredible advancements in IBD treatment, they pose several notable limitations. The use of biological agents and immunomodulators in the management of IBD have been associated with increased viral, mycobacterial, and opportunistic infection. Because many IBD patients receive a multi-drug treatment course, it is often difficult to definitively attribute toxicity to one particular biological agent (D'Haens, 2007). Biological agents that facilitate cell lysis can also increase circulating cellular debris. This can potentially lead to dangerous autoimmunity against key intrinsic cellular components such as nuclear factors and DNA. A 2003 study looking at autoimmunity in Crohn's disease patients treated with infliximab, an anti-TNF agent, found that cumulative incidence of antinuclear antibodies among the study cohort was over 50% after 24 months of treatment (Vermeire et al., 2003). Additionally, biological agents only reduce mucosal inflammation, however, mucosal healing is also an important therapeutic target and predicts clinical remission and resection-free survival in IBD. Besides reduced inflammatory sitimulations, mucosal injuries such as ulcers must be resolved to achieve normal physiological conditions. Biological agents and immunomodulatory therapies do not address the structural pathologies associated with IBD prognosis.

There is no single physiological mechanism associated with the pathology of IBD. Most current IBD therapies only target immunologic modulations during IBD to reduce inflammatory signaling pathways because the pathology of IBD is complex and multifaceted. While the exact

etiology of IBD is unknown, previous research supports the idea that IBD pathogenesis is significantly associated with the dysregulation of the intestinal epithelial barrier to initiate observed pathological and symptomatic conditions. This barrier dysregulation is mediated by several proteins and signaling pathways which may lead to the mucosal inflammation and damage that is characteristic of IBD, making them favorable targets for IBD treatment techniques.

Intestinal Organoids as Models for the Study of IBD

The intestinal epithelium is highly regenerative with many different cell types. The epithelium also requires many villi to assist in absorption and requires motility to carry out mixing, propulsion, and separation of luminal contents. Mechanical components are just as important as chemical components of intestinal cell models. Cells undergo mechanotransduction to sense physical forces and translate them into biological responses, and this function must be translated to in vitro cell cultures to accurately replicate the intestine.

Caco-2 cells are the most widely used cell model and can be used to predict drug intestinal permeability in humans. Caco-2 cells are derived from human colorectal adenocarcinoma, often used in intestinal models, and differentiate within 21 days. However, a pure Caco-2 culture cannot account for important factors such as the mucus layer or interactions between stroma and epithelium. Speeding up culturing or adding in other cell lines such as mucus producing HT29 can improve the complexity of these models. Although other models have attempted to isolate intestinal epithelium directly from human tissue and include growth factors and interaction between epithelial cells and myofibroblasts, these models cannot sustain themselves long term. Finally, since the identification of Lgr5 stem cells, it has become the most

widely used cell line to model “mini gut” organoids that are self sufficient. Despite their many benefits, organoids cannot mimic biochemical forces naturally exposed to stem cells in vivo, and their heterogeneous nature limits drug penetration and permeability. To overcome this challenge, some groups have developed enteroid monolayers organized in 2D, which then neglects the benefits of the 3D model.

To assay intestinal stem cell renewal and differentiation, a self-renewing 2-dimensional primary colonic culture platform is a physiologically relevant system. Previously, three-dimensional organoid culture use was limited due to the inaccessibility of the luminal compartment, as well as was more inaccessible and there were challenges to data gathering data in a three-dimensional matrix. However, long-lived, self-renewing tissue cultured from primary colon cells has not been accomplished. The monolayers formed organoids or colonoids when in matrigel cultures, and the surface matrix and chemical factors that sustain two-dimensional mouse colonic and human rectal epithelial cell monolayers with cell repertoires are comparable to in vivo (Beumer & Clevers, 2016). A two dimensional (2-D) planar tissue construct is a solution that addresses these major challenges and has the potential to further transform *in vitro* study of the gut epithelium (Wang et al., 2017).

Intestinal Stem Cells as a Potential IBD Therapeutic Target

Intestinal renewal and regeneration upon injury is mediated by intestinal epithelial stem cells (ISCs), a cluster of undifferentiated cells located in the intestinal crypt. ISCs have tremendous potential as therapeutic agents for IBD. Several recent studies have developed novel models and methods which utilize ISC to achieve mucosal healing for patients that are resistant to current biological agents. Tissue regeneration of the intestinal epithelial barrier occurs in three

phases: restitution of Intestinal Epithelial Cells (IEC), proliferation of IEC to cover wounds, and reconstitution of tissue to reform crypt structure. The Wnt5 pathway is critical to this process. While there are a multitude of ISCs, bone marrow-derived stem cell transplantation has been found as a safer and more effective method than hematopoietic stem cell transplantation which has also been utilized in IBD therapy (Shimizu, 2019). Stem cells derived from the intestine itself have also been gaining popularity.

Studies have found that certain IEC subpopulations are able to gain plasticity to function as ISCs to contribute to IEC regeneration and tissue repair when other ISCs are eliminated by lethal radiation (Shimizu, 2019). Recently, scientists have successfully overcome the difficulties of isolating and maintaining ISCs and established a novel long-term culture method for ISCs by maintaining them in a 3D-structure, known as “organoids”. This method requires many growth factors, including Wnt3a, R-Spondin-1, EGF, and Noggin (Okamoto, 2020).

Once ISCs are cultured and isolated, they can be used to treat wounds and lesions of the intestinal epithelial barrier in IBD patients. Autologous, endoscopic transplantation of ISCs are a promising application of this strategy for IBD treatment (Okamoto, 2020). ISCs can be collected from the intact lesion of a patient through the endoscopic biopsy, and then expanded *in vitro* by the established organoid culture method. After growing them to a desired number of cells, they can be transplanted onto the target site through an endoscopic delivery method (Okamoto, 2020). It has also been suggested that controlling and minimizing intestinal inflammation prior to transplantation will improve success of transplantation and regeneration. Minimizing inflammation can occur through a number of methods including treatment with specific gut microbiota.

The expected benefits of this transplantation method are improvement in mucosal healing rate and possible reduction of the risk in developing colitis-associated cancer (Okamoto, 2020). These cancers often arise from genetic and epigenetic changes within the ISCs through their long-term exposure to the inflammatory environment. Replacing exhausted ISCs experiencing this long term exposure with new ISCs that were grown in the most ideal and stable environment could potentially reduce the initiation of colitis-associated cancers (Okamoto, 2020). In one clinical study utilizing this therapeutic treatment method, organoids were developed from biopsy samples of IBD patients and resulted in ISC transplantation on the wound bed of patients. Through the process of IEC restitution and proliferation, these transplanted stem cells could fully cover and heal the wound, reduce inflammation, and re-establish normal barrier function (Shimizu, 2019).

Application of Intestinal Organoids for Gastrointestinal Disorders

The unique ability for 3D organoid systems to self-organize have rendered them highly similar to real human organs(Serra et al., 2019). Their similarities have made using 3D organoids particularly advantageous models for studying the pathology and manifestation of gastrointestinal disease. They can also be developed and maintained more easily than live organism models and can be easily genetically manipulated to understand various genetic factors underlying disease development(Fujii et al., 2015). The organoid model can also reduce experimental animal use because of their similarity to *in vivo* tissue. Intestinal organoids have been used to successfully model the pathology of diseases such inflammatory bowel disease and cystic fibrosis, and have proven promising in serving as tools to better understand the pathogenesis of various digestive disorders(Dekkers et al., 2013; Xu et al., 2021).

Usage of Intestinal Organoids in the Inflammatory Bowel Disease Research

Inflammatory Bowel Disease (IBD) is characterized by mucosal inflammation of the GI tract that is caused by a combination of genetic susceptibility, immune dysregulation, disruptions in the microbial communities, and environmental factors(De Mattos et al., 2015). Most forms of IBD are polygenic, with multiple susceptibility loci contributing to the overall risk of disease(Loddo et al,. 2015). While there are a number of possible underlying physiological mechanisms that can lead to IBD, all mechanisms involve the dysregulation of the intestinal epithelial barrier to initiate the pathological conditions. The complexity of the disease's pathology makes developing treatment techniques difficult, so studying IBD and finding novel ways to model the pathogenesis of the disease is increasingly urgent. Because the mechanisms of IBD pathogenesis are influenced by a number of variables including genetic, immune dysregulation, and environmental factors, accounting for these complexities is difficult in an *in vitro* setting. Most conventional treatments, including recently developed biologic agents, target only the immunologic modulations during IBD to reduce inflammatory signaling pathways (Moreno et al,. 2021). However, mucosal healing is also an important therapeutic target in IBD (Rutgeerts et al,. 2007).

While mucosal healing as a therapeutic target for IBD is promising, the development of IBD treatments targeting mucosal healing has also been slowed by a lack of long-lasting human intestinal culture models(Yoo et al., 2019). Organoid cultures have been used to study a variety of different cellular pathways as well as how different factors affect the overall integrity of the intestinal epithelium and their regeneration process(Dedhia et al., 2016). A 2014 Grabinger et al. explored the potential of using intestinal crypt organoids *in vivo* to model intestinal epithelial

cell death, which they hypothesized could be scaled to understand how various toxins and drugs that damage the intestinal epithelium are implicated in the cell death pathway(Grabinger et al,. 2014). Organoids were exposed to tumor necrosis factor-alpha (TNFa), cisplatin, and UV irradiation to induce apoptosis. Inducing cell death using the following triggers resulted in the disrupted integrity of the organoids, including epithelial cell death and a disintegrated epithelium. Recent studies have also successfully developed organoid cultures from intestinal crypts isolated from IBD, demonstrating that these organoids have the potential to be used as therapeutic drug-testing models for epithelial healing in IBD cases.

Limitations of Using Organoids to Model GI Disease

Despite the importance of organoids in modeling disease pathology, there are also a number of limitations. One key limitation is the use of Matrigel, a heterogeneous modeling matrix containing a number of ill-defined proteins(Ainsenbrey et al,. 2021). The ambiguous definition of Matrigel may lead to uncertainty in terms of how the Matrigel interacts with the human organoid model when studying the pathophysiology of gastrointestinal disease. Matrigel is also an animal-derived matrix, which may distort the integrity of studying human disease pathophysiology. Although there are synthetic hydrogel alternatives to matrigel when establishing human organoids, these hydrogels are relatively new, and Matrigel is the most common extracellular matrix used in organoid development.

Organoids are isolated models of various organ systems, which may make it difficult to account for the complex relationships organs of interest have with their surrounding environment and other organ systems. The gastrointestinal tract is a complex system, which includes an interactive environment consisting of immune cells, stromal cells, neuronal cells,

and the microbiota etc(Johnson et al., 2006). These populations interact with each other to maintain homeostasis and can contribute to the pathogenesis of diseases and their resolution. It is almost impossible to mimic this complexity in intestinal organoids models. More generally, there is a great variability in the organoids isolated from different patients, even those with the same condition. Considering this, Matrigel with its variability contributes as a confounding variable. Evidently, extrapolating results from intestinal organoids is challenging. Thus, it is important to simplify your experiments, reducing experimental noise is vital for making one's experiments easier for interpretation.

Experimental Basis for Our Research

The Wnt signaling pathway is responsible for the proliferation and differentiation of these ISCs and is regulated by β-catenin proteins found in adherens junction complexes. The β-catenin in this signaling pathway ultimately leads to the regeneration of the intestinal barrier during inflammation caused by common disease pathologies such as IBD (Garcia et al., 2018). β-catenin activity is further regulated by E-cadherin, an adherens junction protein that plays a central role in both maintaining the structural integrity of the intestinal barrier and maturing paneth and goblet cells involved in the intestine's innate immune response (Schneider et al., 2010). E-cadherin serves as a protein anchor for β-catenin, which facilitates cell to cell adhesion and homeostasis. Reduced E-cadherin during injury, particularly in the pathology of IBD, increased release of β-catenin and ultimately promoted proliferation of epithelial cells.

Egtazic acid, or EGTA, can mimic the disruption of E-cadherin binding to β-catenin. E-cadherin depends on calcium ions to establish cell-to-cell contact. By introducing a calcium chelating agent such EGTA, E-cadherin becomes more susceptible to protease activity and is

unable to anchor β-catenin in the adherens junctions (Nagar et al., 1996). With more unbound β-catenin in the cytosol, the β-catenin goes to the nucleus and increases transcriptional activity which results in increased ISC proliferation. The activation of the Wnt/β-catenin signaling is essential during intestinal homeostasis and regeneration. Understanding the impact of E-cadherin on intestinal epithelial populations and their contributions to mucosal regeneration is critical to developing a modular therapeutic target for IBD.

Our Research Question and Hypothesis

Our research focused on characterizing the role of adherens junction proteins, specifically E-cadherin, in a variety of understudied physiological contexts. Understanding the role E-cadherin plays during the dysregulation and recovery of the intestinal epithelial barrier can provide a critical lens into the use of these signaling pathways as promising targets for novel IBD therapeutics. First, we looked to define the role of adherens junctions during the development of enteroid and colonoid models. We hypothesized that E-cadherin disruption will release β-catenin from adherens junctions, resulting in increased Wnt signaling pathway activity and subsequently increased proliferation of ISCs. Our research framework and methodology also looked to define the role of adherens junction proteins in Wnt pathway signaling in distinct ISCs during homeostasis and colitis. While this portion of the project was ultimately not completed by our team, it remains an important component of our research methodology. Together, these research aims cast a wide net in filling the gaps present in our target body of knowledge.

The experimental design of this research is carried out in three portions. The first portion is to maintain the 3D mouse colonoids to be manipulated and used as a model organism for intestinal epithelial tissue. The second part of our method includes inducing injury via EGTA

(broad calcium chelator) and BAPTA (extracellular calcium chelator) solutions composed of differing concentrations. The final portion of experimentation was the analysis portion which was done through microscopy and visual observations. Prior to the experiment, the supply of L-WRN conditioned media was being continually replenished to sustain the colonoids culture models. L-WRN CM consists of Wnt-3a, R-spondin 3, and Noggin and is required for the intestinal stem cell niche.

Chapter 2: Materials and Methods

Maintaining the Colonoids Culture

The colonoid media was made first. The colonoid media was produced by creating L-WRN conditioned media. This media was made by engineering L line cells that would produce the Wnt R spondin and noggin proteins that are needed for healthy epithelial cell growth in the intestine. The media of these cells was then collected about every day, and that media was used to culture the murine GI cells. This conditioned media had additional antibiotics and factors added and was used to treat the colonoids. The 3D mouse colonoids were used for the experiments, and two 48 well plates were used in total. To passage the mouse intestinal Organoids, the liquid culture medium from each of the wells was then removed to be passaged without disturbing the dome of organoids in Matrigel. 1 mL Gentle Cell Dissociation Reagent was then added on top of the exposed dome in each well and incubated at room temperature for one minute a rinse A 1 mL pipette tip was pre-wet with the Gentle Cell Dissociation Reagent in the well and used to break up the dome and organoids by pipetting the 1 mL liquid in the well up and down approximately 20 times. The same pipette tip was used to transfer the suspension to a 15 mL conical tube. The culture well was then washed with an additional 1 mL Gentle Cell

Dissociation Reagent and added to the tube. This process was done for each potential well, and the 15mL tubes were then incubated at room temperature on a rocking platform at 20rpm for 10 minutes. After incubation, tubes were centrifuged at 290xg and 2-8 C for five minutes, then poured off gently and the supernatant was discarded. Pellets were then washed by resuspension in 10mL DMEM/F12, and centrifuged at 200xg at 2-8C for five minutes. Supernatant was then aspirated off. 100 µL/48 well room temperature colonoid medium was added to the pellet in each 5 ml media tube, as well as 100 µL/4 well undiluted Matrigel. This mixture was then seeded into 96-well plates and allowed to grow for up to 5 days before beginning treatment with EGTA.

EGTA-Induced E-Cadherin Disruption

In order to make the EGTA concentrations needed for our experimental design, we first measured out a mass of EGTA that was diluted in NaOH in order to get a 0.5M EGTA molar concentration, The mixture was then vortexed until the solution was uniform, and placed in a bead bath as needed to insure dissolution. In order to create serial dilutions of the 1.4 mM, 1.2 mM, 1.0 mM, 0.8 mM, 0.6 mM EGTA, and 1.4 mM and 1.0 mM NaOH for the vehicle condition, we used serial dilution calculations to add appropriate amounts of NaOH or 0.5M stock concentration EGTA uL to tube filled tubes with 1mL of colonoid media preheated in a bead bath. Wells were treated starting on Day 5 after passaging before cell differentiation with the created media solutions every two days. This was done through aspiration of media in each of the 96 wells, while avoiding damage to the matrigel attached to the well. After careful and thorough aspiration, 40uL of solutions were added to each well and changed every other day.

Data Analysis

Pictures of the 48 well plates were taken daily using a Nikon SMZ 745T light microscope. Observations and treatment continued until Day 10. In terms of morphological Analysis, ImageJ was used to quantitatively analyze the area of colonoids to evaluate the organoid growth and proliferation, and one way ANOVA was run on Minitab on the area versus treatment with a null hypothesis of equal means and a 95% confidence interval while utilizing a Tukey, Fisher, and Dunnett test.

Chapter 3: Results

The following images are representative images of the colonoids cultured in the 96-well plate:

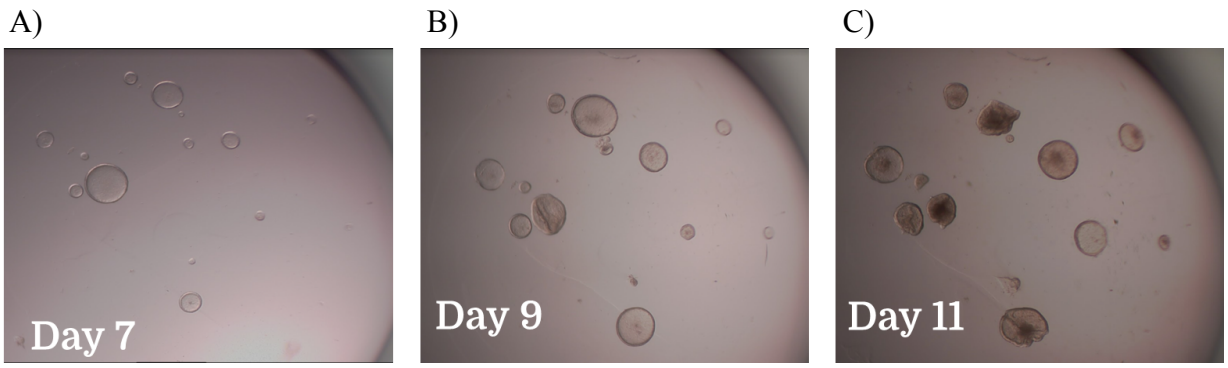

Figure 1a., 1b., 1c.: 3D colonoids cultured on Matrigel in a 96-well plate. Cells were treated every day starting on Day 1 with EGTA at varying concentrations or vehicle control. Colonoid growth on day 7, 9, and 11 are displayed.

Comparative analysis was performed to assess the effects of culturing colonoids with L-WRN conditioned media only, L-WRN CM with NaOH (the vehicle for EGTA treatment), and L-WRN CM with 1mM EGTA. Representative images of each treatment condition are displayed below:

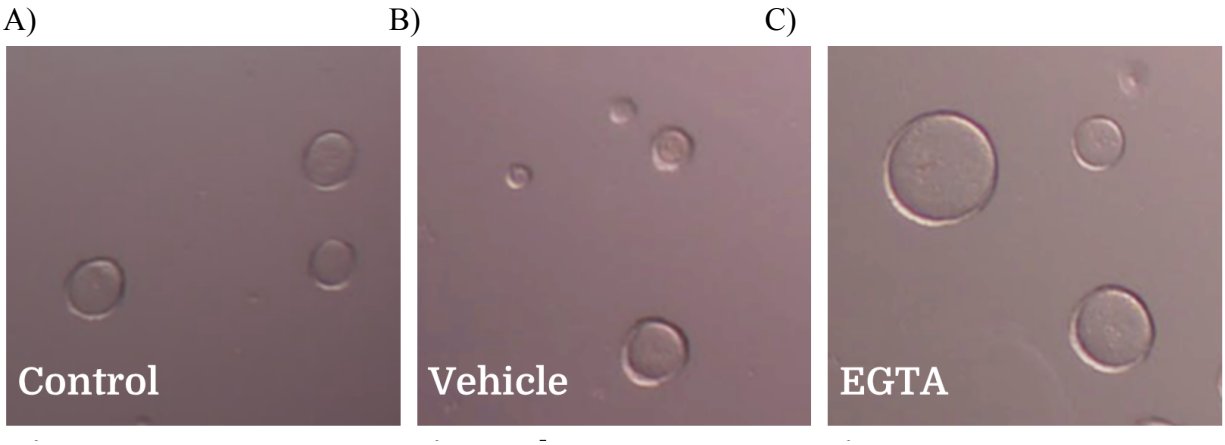

Figure 2a., 2b., 2c.: Colonoids grown 6-days post seeding in each treatment condition. A) Colonoids grown with only L-WRN conditioned media. B) Colonoids grown with L-WRN CM and NaOH. C) Colonoids grown with L-WRN CM and 1mM EGTA.

Using ImageJ, the area of colonoids from 4 wells in each treatment condition were assessed over time. Images of colonoids used for analysis were taken on day 6, 7, and 8 post-seeding. The area of every colonoid imaged in each well was measured, and the average area and standard error was calculated for each well. Finally, the average of all 4 wells for each treatment condition was taken and plotted over the three days. 33 colonoids were assessed for L-WRN CM, 34 colonoids were assessed for L-WRN CM + NaOH, and 43 colonoids were assessed for L-WRN CM + 1mM EGTA. The results of the analysis are tabled below:

Table 1: Average area of colonoids grown in each treatment condition on day 6, 7, and 8.

	Treatment Condition		
Day	**L-WRN CM only**	**Vehicle (L-WRN CM + NaOH)**	**L-WRN CM + 1mM EGTA**
6	0.0155 mm^2 (SE: 0.0026 mm^2)	0.0091 mm^2 (SE: 0.0011 mm^2)	0.0190 mm^2 (SE: 0.0056 mm^2)
7	0.0225 mm^2 (SE:	0.0320 mm^2 (SE:	0.0323 mm^2 (SE:

	0.0037 mm^2)	0.0048 mm^2)	0.0063 mm^2)
8	0.0439 mm^2 (SE: 0.0071 mm^2)	0.0541 mm^2 (SE: 0.0090 mm^2)	0.0604 mm^2 (SE: 0.0104 mm^2)

These results indicate that colonoids treated with EGTA have increased growth compared to those treated with L-WRN CM only or L-WRN CM + NaOH vehicle. EGTA may induce increased proliferation of colonoids via upregulation of WNT signaling.

The results of this experiment are plotted below in figure 3:

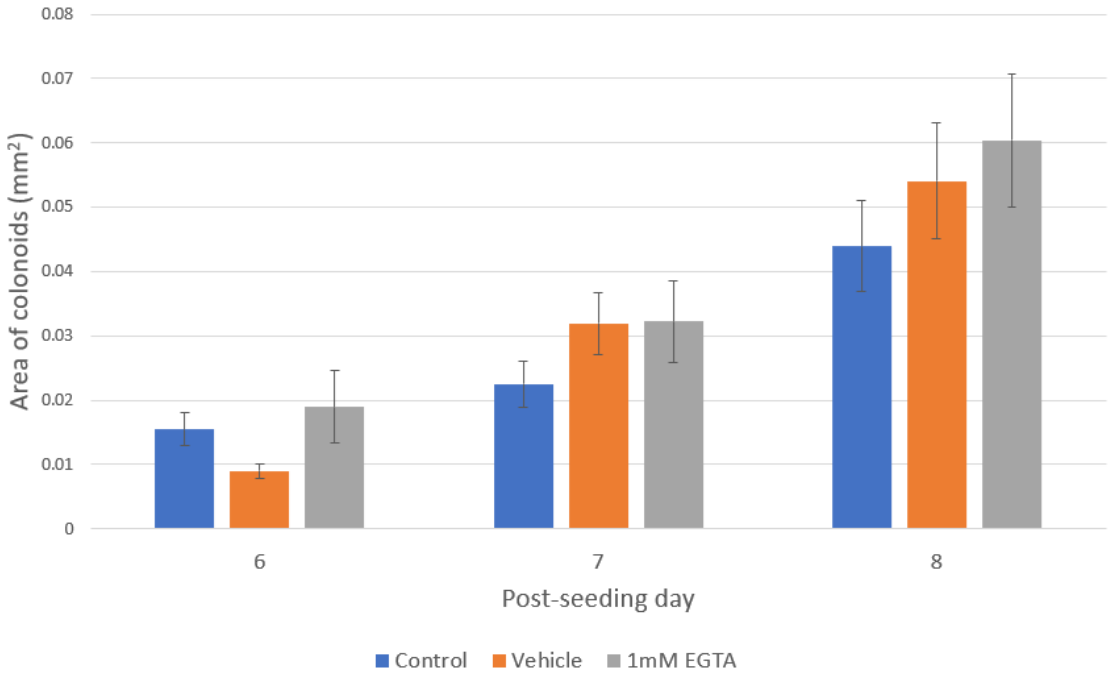

Figure 3.: Graphed colonoid area on day 6, 7, and 8 post-seeding for each treatment group. Colonoid area for each treatment group was analyzed and averaged over four wells for each day. EGTA treated colonoids had the largest average colonoid size from days 6-8.

Through the multiple experiments our team conducted using EGTA to treat our 3D organoids, we measured and observed the morphological changes to the organoids. Through experiments assessing the effect of different concentrations of EGTA use on organoid

morphology, we found that use of 1 mM EGTA induced an increase in the area of colonoids while not causing excess cell damage or death. Additionally, morphologic observation showed that EGTA-induced E-cadherin disruption resulted in increased growth of the colonoid.

Chapter Four: Discussion

The colonoids treated with EGTA tended to have slightly larger colonoids than those of the vehicle control. Although this difference is not statistically significant, we suggest EGTA may induce increased proliferation of colonoids via WNT upregulation . A repeat validation experiment is necessary in which we will assess Wnt signaling levels and characterize the intestinal cell population by qPCR and immunofluorescence.

For future studies, we will study proliferation using Ki67 staining and PI staining methods with or without treatment of EGTA. Additionally, we will aim to demonstrate the EGTA-induced disruption of E-cadherin and increased cytosolic/nuclear β-catenin using IF microscope analysis to further confirm our current data and hypotheses. We would also like to explore the significance of the Wnt signaling pathways and different cellular populations using qPCR and IF microscope analysis.

Our study has clinical applications for patients living with IBD. Using regenerative medicine, one can harvest cells of IBD patients and culture in vitro organoids, temporarily downregulate E-cadherin, and transplant them back into the patients. These organoids once implanted would rejuvenate and regenerate the inflamed intestinal tract, making it a viable clinical therapeutic for those who are resistant to traditional treatments. Ultimately, we can apply this to a clinical approach to address IBD in patients resistant to traditional treatments.

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