ABSTRACT

BOGER, KASEY DAYLE. Establishment and Characterization of the Intestinal Porcine Jejunal Cell Line (IPEC-J2) Using Wnt-based Crypt Culture Media. (Under the direction of Dr. Anthony Blikslager)

The intestinal epithelial barrier is susceptible to injury from insults such as ischemia or infectious disease. The epithelium's ability to repair wounded regions is critical to maintaining barrier integrity. Mechanisms of intestinal epithelial repair can be studied with models that recapitulate the *in vivo* environment, which is the focus of Chapter 1. This chapter contains a literature review on *in vitro* injury models and intestinal cell lines utilized in such systems and summarizes the advantages of these systems while also illustrating remaining areas for improvement in intestinal *in vitro* modeling. Specifically, this review discusses the lack of optimal intestinal epithelial cell lines to study intestinal physiologic properties such as barrier function, and reparative processes. The lack of relevant cell lines has also hindered research regarding intestinal disease pathogenesis. Currently, the IPEC-J2 cell line is a non-transformed porcine jejunal cell line utilized as an *in vitro* model of intestinal epithelium, but it has shown atypically high measures of intestinal barrier function (transepithelial electrical resistance [TEER]) and a lack of heterogeneity in cell types to accurately represent native tissue-like conditions, making its practical applications to gastrointestinal physiology questionable. Chapter 2 focuses on characterizing the IPEC-J2 cell line in medium including Wnt, Noggin, and R-spondin (WRN), factors known to be present in the crypt base *in vivo*. The IPEC-J2 cells supplemented with WRN and grown to confluence had lower TEER (47.1 ± 1.8 $\Omega \bullet cm^2$) than confluent IPEC-J2 cells grown in conventional cell culture media (1,856 ± 109.2 $\Omega \bullet cm^2$) after 10 days, and were more similar to neonatal (47.8 ± 6.9 $\Omega \bullet cm^2$) and juvenile porcine jejunal tissues (41.0 ± 2.6 $\Omega \bullet cm^2$). In addition, the IPEC-J2/WRN cells contained a larger stem cell and enteroendocrine cell populations, as well

as increased evidence of goblet cells and enterocytes, showing greater heterogeneity than IPEC-J2 cells in conventional culture, and more representative of the populations of cells seen in native tissues. IPEC-J2/WRN cells appear to be a more physiological *in vitro* model for the study of intestinal pathophysiology. Furthermore, this model was successfully utilized in the study of a novel drug for Celiac disease and holds promise as an improved model for the in vitro study of intestinal pathophysiology.

Establishment and Characterization of the Intestinal Porcine Jejunal Cell Line (IPEC-J2) Using Crypt Culture Media

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DEDICATION

This thesis is dedicated to my friends and family. Thank you for your unending support and for pushing me to achieve all that I set out to accomplish. To my husband, Nick, I am especially grateful for all of your love and commitment and I would not want to pursue my goals in life with anyone else by my side.

BIOGRAPHY

Kasey Boger was born in Hickory, North Carolina. She attended North Carolina State University and obtained a Bachelor of Science degree in Biological Sciences in 2018. In 2019, she began pursuing her Masters degree in Comparative Biomedical Sciences under the direction of Dr. Anthony Blikslager in the Comparative Gastroenterology Lab. Kasey aspires to become a small animal veterinarian in the future.

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CHAPTER 1

Mechanisms and Modeling of Wound Repair in the Intestinal Epithelium

Introduction

The intestinal mucosa is lined by a single layer of epithelium that serves as a crucial barrier between the gut lumen and the body. Numerous insults are known to disrupt the epithelial barrier. For example, SARS-CoV2 (also known as COVID-19) has profound effects on the intestinal epithelium. This is believed to be in part due to the affinity SARS-CoV2 S for the angiotensin converting enzyme 2 receptors (ACE2) which is extensively expressed on the apical surface of small intestinal epithelium. While the exact mechanism is not entirely clear, the ability of SARS-CoV2 to damage the intestinal mucosa and result in increased permeability may result in the onset of diarrhea and malabsorption [1]. Numerous other infectious diseases, such as Rotavirus, also disrupt epithelial barrier function, and disease processes such as ischemia/ reperfusion are known to result in epithelial lifting from the basement membrane, and loss of epithelial monolayer integrity [2-6]. Therefore, a crucial component of epithelial barrier function is its ability to rapidly repair using a mechanism called restitution. This involves cell spreading and extension of lamellipodia in order to bridge the wound that results from sloughing of epithelium [7] Reductionist models of restitution have been employed to develop a mechanistic understanding of restitution, including *in vitro* cell models. However, restitution remains incompletely understood, and this is partly related to the anatomical and physiological complexity of the intestinal mucosa and the limitations of cell models and methods of this reductionist barrier modeling. The wounding process itself can also present a technical dilemma. For instance, wounding typically involves 'scratching' in a mechanical fashion, but this differs from *in vivo* wounding more typically attributable to inflammation from infectious disease or hypoxia and inflammation induced by ischemia/ reperfusion. Therefore, this review outlines differing models of epithelial wounding and

the characteristics of the cell lines used to aid investigators in designing optimal epithelial wounding models for the study of barrier restitution.

The Intestinal Barrier

The epithelial barrier functions as a physical boundary between luminal contents and the remainder of the body while also serving as a filter for crucial nutrients, ions, and water. Effective barrier function is therefore essential to maintaining intestinal homeostasis and this should be recapitulated in *in vitro* intestinal modeling systems. Tight junctional proteins bridge across the paracellular space of subjacent epithelial cells, and are largely responsible for controlling the flow of ions and macromolecules between cells and create measurable differences in transepithelial electrical resistance (TEER) and macromolecular flux. However, tight junctional proteins retain their own unique size-selective properties which are classified as either pore or leak pathways. Pore pathways are defined as those permeable to molecules with a radius of ~4 Å or less, whereas leak pathways are defined as allowing the passage of large non-charged solutes [8]. To accurately assess the integrity of these pathways, methods to assess TEER have been developed for use in cells. One pitfall of using TEER to assess wound healing is that both transcellular and paracellular transport contribute to this measurement, whereas macromolecular fluxes of labeled macromolecules, such as FITC-dextran, are more indicative of changes to the paracellular space [9]. Therefore, to avoid confusion as to whether cellular crawling or closure of tight junctions is responsible for differences in barrier function, restitution studies continue to largely rely on area of epithelial wound coverage.

Mechanisms of barrier repair

In the small intestine, following barrier injury there are three phases of wound healing: villus contraction, cell migration (restitution), and paracellular space closure [7]. Given that cell migration is a major component of the reparative response to injury, different wound models have been developed to closely examine this process during the restitution of epithelial barrier defects. Epithelial restitution utilizes the ability of epithelial cells adjacent to injured portions of mucosa to flatten and migrate over the intact basement membrane following epithelial loss attributable to insults such as infectious disease, or ischemia [10]. This process is accomplished when cell-cell and cell-matrix adhesions are temporarily remodeled and allow for cells to extend forward and cover denuded surfaces, and can occur rapidly before the onset of increased enterocyte proliferation to ultimately replace lost epithelial cells.

Originally, investigators focused on the mechanisms that initiate restitution. One pivotal study in epithelial restitution performed by Nusrat *et al* evaluated cellular migration while cell monolayers recovered from scratch wounding *in vitro*. The study ultimately concluded that cell flattening, spreading and formation of lamellipodia-like protrusions were among the first steps involved in restitution [11] . However, a greater understanding of mechanisms of restitution is needed to screen new potential therapeutics and extend findings to the clinic. One study utilized IEC-6 cells in order to evaluate the effects of various cytokines on wound closure, and found enhanced epithelial restitution when cells were exposed to TGF-α, EGF, and IL-1β whereas there was no change in migration of cells surrounding wounds in the presence of IL-6, TNF-α, PDGF, and lipopolysaccharide [12]. Raising questions about the role of inflammatory cytokines in restitution, IFN-γ has been found to have an inhibitory effect on restitution as a result of dysfunction in the F-actin lamellipodia on T84 cells at the edge of epithelial woundsin contrast to

findings with other cytokines [13]. Other studies have focused on the role of hypoxic conditions at the site of mucosal injury. For example, activation of hypoxia-inducible factor-1α (HIF-1α) has been found to play a role in epithelial wound repair [14-16]. Specifically, one study found an increase in expression of fibroblast integrin β1 (ITGB1) through transcriptional mechanisms dependent on HIF which is critical to epithelial wound restitution [17]. However, finite mechanisms of restitution remain to be fully characterized. Therefore, the restitution process continues to be studied extensively using scratch wounding models, cell exclusion assays, neutrophil-induced epithelial wounding models, and electrical wound-healing assays to study healing events, and the mechanisms that regulate cell crawling.

Following restitution, wound healing is completed by resealing of tight junctions. Select tight junction proteins have been studied to refine our understanding of reparative mechanisms of tight junctions. For example, claudins have been investigated and found to have seemingly complementary functions in barrier function, with some claudins acting as "leaky" or pore-forming claudins, and others as "tight" sealing claudins [18]. In wound healing studies assessing the role of ClC-2 in ischemic-injured murine jejunum, a critical role for claudin-1 and occludin in resealing tight junctions following epithelial restitution was demonstrated to be the final step in normalizing barrier function [19]. Additionally, an *in vitro* study using Caco-2 cells overexpressing ClC-2 resulted in a decrease of the pore-forming claudin-2 protein while maintaining claudin-1 and claudin-4 expression, thereby enhancing barrier function [20]. Other tight junction proteins have been studied as it appears their reassembly plays a role in epithelial repair; however further investigation is needed to fully understand the extent of their role in healing.

In Vitro Injury Models

A number of in vitro cellular wounding and repair models have been created to assess epithelial restitution, all of which have strengths and weaknesses when attempting to recapitulate intestinal wound repair in clinical patients. In addition, models to explore the more complex effects of multiple cell types on wound repair have also been studied, particularly the role of epithelial neutrophil transmigration, in order to provide more physiologically relevant models.

Scratch Assay

Scratch wounding is one of the most frequently utilized methods to study barrier restitution *in vitro*. It offers a simple procedure to induce cell crawling similar to that which happens *in vivo* post injury. Due to its simplicity, it can be used on any cell line or primary culture that can successfully be grown into a monolayer. This method involves growing confluent monolayers, scratch wounding, and wound measurement. Scratch processes differ by lab, but the two most popular methods are scratches formed by razor blades and those created by pipette tips [21-31]. Typically, razor blades are carefully pressed into the confluent monolayer of cells while pipette tips are dragged across it (Figure 1). More advanced methods of scratching have been developed to standardize the scratch. One technique resembles a multichannel pipette, but has guiding rails on the outside to line up with the plate. This method scratches 8 wells at once and has adjustable hex screws to set varying angles [32]. Another method does not use any pipette tips, but instead uses a 96 floating-pin array. The operator sets the device on top of the plate, engages the pins, and drags the device towards one corner of the plate scratching 96 wells at once [33]. These advancements have helped to standardize scratches within experiments.

Initially, cells are grown to confluence, after which monolayers are either serum deprived or serum reduced from anywhere between 18-48 hours before the wound is inflicted. The level of serum present depends on the type of cell. Transformed cell lines can accommodate complete serum deprivation while primary cells continue to need small amounts of serum to function properly [34]. The intention of serum deprivation is to coerce the cells into a quiescent state, so that cell proliferation is not mistaken for cell migration. Although serum deprivation is a standard step in many scratch wound assays, serum deprivation is somewhat controversial. In some cell lines, it can cause unpredictable effects such as phenotypic changes or varying responses to certain stimuli [35]. Given this controversy, researchers need to decide whether serum deprivation or reduction is a necessary component of their scratch wound assay in order to optimally study restitution. In addition, proliferation assays like nuclear labeling with Edu incorporation may be used to ensure rapid proliferation into the wound bed is not being misinterpreted as purely cellular migration [36]. Edu labeling has been utilized in place of BrdU as it uses a simple chemical reaction instead of DNA denaturization in order to analyze cellular proliferation, population homeostasis, and cell marking procedures [37].

Once the monolayers are injured, the wells can be washed to ensure there are no remaining cells in the scratched area. Immediately following this, the cells and the scratch are imaged to assess the size of the wound. The wells are then imaged at different time intervals to record the migration process. Quantification of wound closure can also vary; Some researchers choose to do a simple count of nucleated cells across the wound margins [24, 25, 27, 28], while others use image analysis software to compare the area of the scratch before, during, and after cell migration [7, 22, 26, 38, 39]. Overall, the process of scratch assays is relatively straightforward and does not require advanced technology.

Due to the simplicity of this process, it has been used for many animal cell line studies to determine whether different growth factors [23, 25, 38, 40], cytokines [27], mRNA proteins [26], nucleic acids [28, 39], or supplementations [22, 24] improve the epithelial restitution by enhancing cell migration. For example, when TGF-β was added to medium, although it is known for the inhibition of proliferation, it actually sped up the migration process post scratching [25].

Although scratch wounding is both quick and simple, it does have its drawbacks. In order for comparison among studies and among samples to be made, the scratches need to be of similar length, width, and depth. There is extensive variability between scratching tools and techniques. Even within the same procedure, variation occurs due to varying operator dexterity patterns (pressure, angle, length) between each scratch. However, additional tools previously mentioned have been developed to minimize these discrepancies. Another problem with scratch assays is that the scratching instrument disrupts more than just the cells. Specifically, most experiments require culture surfaces to be coated with components of the extracellular matrix (ECM). This matrix is often also damaged during wound infliction. This damage can affect the migration of the cells and adds yet another variable impacting restitution [41]. In order for scratch assays to be compared, standard procedures including scratch methods and coating materials should be established.

Cell Exclusion Zone Assay

Given its injurious nature to epithelial cells, the scratch wounding method has a multitude of limitations that may influence restitution such as an influx of intracellular fluid or ECM damage [42]. Therefore, the cell exclusion zone assay was created to analyze cell crawling in response solely to neighboring barren space, thereby eliminating some of the confounding factors generated by physically wounding a monolayer. Cell exclusion models use patterned cell seeding, created by

molds that are placed on the surface before the cells are seeded (Figure 1). Before this assay was even named, the concept was first used in 1984 to study cytoskeletal regulation in aortic endothelial cells [43]. In that experiment, a Teflon fence was used to create the cell barren areas. The experiment that coined the name 'Cell Exclusion Zone Assay' used a microfabricated Polydimethylsiloxane (PDMS) mold with rectangular holes in it [42]. The mold was placed on ECM coated plastic, and cells were seeded into the holes. The cells were grown to confluence, and the molds were peeled off. This resulted in geometrically perfect 'wounds' into which the cells began crawling. The migration rate or area coverage was captured microscopically and then analyzed. This method has been utilized amongst a wide variety of epithelial cell crawling studies involving kidney cells [42], esophageal cells [44], and bronchial cells [45]. Due to the fast-healing effects of the intestine, this assay was also optimized in the intestine. In this study, researchers employed the cell exclusion zone model to study cell crawling of fetal small intestine (FHs-74 int) cells [46]. Silicone stoppers were used to create the cell barren spaces, after which cell crawling was microscopically examined. This model was compared to the standard scratch wounding assays, with the key finding that the cell exclusion zone assay created a more uniform wound with no damage to the underlying ECM components or leakage of intracellular contents as would occur during scratching, making the quantification of cell crawling easier and more accurate [46].

However, this model does not perfectly recreate *in vivo* wound healing. Researchers have been hesitant to call this cell excluded 'wound' an actual wound because of the non-injurious way that it is created. Nevertheless, this model is still thought to be relevant because cell migration is one of the biggest components of epithelial restitution, and this assay has helped to narrow down mechanisms of cell crawling. For example, researchers using a cell exclusion zone assay with canine kidney cells verified that the cells did not need to be damaged in order to migrate. They

found that exposing the cells to barren space was enough to trigger their movement. This finding supports the hypothesis that there is a mechanical type of communication between cells and exposed matrix which induces migration [42].

Neutrophil wounding models

Intestinal disease processes such as ischemia/ reperfusion injury, inflammatory bowel disease, and infectious disease that damages intestinal epithelium, are all known to attract significant numbers of neutrophils to the sites of epithelial injury [6, 47]. This is in part due to neutrophilic chemoattractants accumulating in the area when oxidants are released by the damaged tissue. For example, LTB_4 is generated from oxidation of lipid membranes, and is a potent neutrophil chemoattractant. As neutrophils are chemoattracted to the basal side of epithelium via chemoattractant gradients, they continue to migrate through paracellular pathways, disrupting interepithelial junctional structures, ultimately entering the intestinal lumen. This process is regulated in part by CD44v6 and CD55 as well as ICAM-1 [48]. As the number of neutrophils migrating across the epithelium increases, paracellular spaces are initially widened, followed by lifting and loss of epithelium, with wound formation [49].

To model this process *in vitro,* advances in existing cell motility models have occurred to observe neutrophilic migration across epithelial monolayers. Notably, Brazil et al. developed an advanced model for investigating mediators associated with increased neutrophil transmigration and luminal migration. To achieve this, T84 monolayers were grown on collagen-coated transwells and a chemotactic gradient was created by adding 100nM formyl-methionyl-leucyl-phenylalanine to the media on the apical side of a cell monolayer in order to establish a basolateral to apical direction of migration. Next, $1x10^6$ polymorphonuclear leukocytes (PMN;

(neutrophils) were added to the upper chambers of transwells (in order to achieve technical simplicity and accuracy, application of neutrophils to the apical surface was adopted) and transmigrated PMNs were measured by a colorimetric enzyme activity assay specific for the PMN azurophilic marker myeloperoxidase (MPO). The resulting round wound was observable by using the epithelial desmosomal marker, desmoglein. [50]. This technique has been further employed to investigate the role of interepithelial junctional complexes in signaling and guiding neutrophil transmigration. For example, a study evaluating junctional-adhesion molecule like protein (JAML) found that binding of JAML to epithelial coxsackie and adenovirus receptor (CAR) plays a role in the regulation of neutrophil transmigration. The bound JAML/CAR fusion proteins specifically inhibited neutrophil migration when introduced into PMN-rich T84 monolayers. While this model can be used to represent wounding following transmigration of PMNs, the restitution that follows is markedly different than that described in other monolayer wound assays. In particular, the wound develops a rim of actinomyosin that appears to help close the wound by contraction, rather than relying solely on epithelial migration[51].

Electric Cell-substrate Impedance Sensing

Modern advances have sought to alleviate the problems of inconsistency noted in scratch wounding systems of modeling. This has led to the development of *in vitro* systems which are able to monitor monolayer behavior after wounding the epithelial cells via high electrical current pulses. The electric cell-substrate impedance sensing (ECIS) system utilizes gold film electrodes which are inserted into the bottom of cell culture plates [52]. Cell monolayers are then established and grown on the surface of this gold film electrode, with a larger counter electrode inserted into the culture media in order to create an electrical circuit. In addition to wounding the cell monolayer, the electrode is then able to quantify and chart changes in impedance resulting from cellular

behavior as they migrate to heal the wound [53]. This technique allows for the investigator to manipulate the wound severity by altering the duration and current strength to fit study parameters.

One study seeking to understand the connection between integrins and epithelial injury in the intestine employed ECIS to help better understand the role of epithelial CD98 in colonic inflammation. This group injured Caco2-BBE cell monolayers apically with application of 3% Dextran Sodium Sulfate (DSS) treatment prior to ECIS with voltage pulses of 40 kHz and 4.5 V for 30s and then allowed the wound to heal. The resulting examination of CD98 during wound infliction and restitution revealed that upregulation of epithelial CD98 mediated by INF-γ during intestinal inflammation in DSS-treated monolayers hampered cellular restitution in this model [54]. Another study performed with Caco2-BBE cell monolayers explored the role of ADAM-15 in intestinal wound healing. Monolayers with overexpressed ADAM-15 and control groups were injured with a voltage pulse of 40Hz and 4.5-V amplitude for 30s to cause cell death. Ultimately, cells with upregulated ADAM-15 did not recover as well as the control group, which was able to completely heal the wound after 19hrs [55].

Where many of the existing models fail to provide consistent injury that can be modified to exacerbate or change the level of injury, ECIS produces a consistent injury via gold film electrodes while also allowing investigators to track restitution in real-time via impedance measurements. However, the heat created from the high current can alter viability of the surrounding cells and cause additional damage. In addition, the density of cell monolayers and the extent to which interepithelial junctions are intact can influence impedance measurements and make interpretation of study results challenging.

Organoid models

Recent advances in 3D organoid models of the intestine have created a unique opportunity to evaluate epithelial injury and repair. Organoids display architectural and physiological similarity to native tissues, including a crypt-villus axis with a stem cell niche within the crypts [56-58]. One study subjected small intestinal organoids to γ-radiation to support previous reports indicating hepatocyte nuclear factor 4α as an upstream regulator of repair [59]. Another investigated stem cell responsiveness in crypt culture, identifying valproic acid and the EZH2 inhibitor EPZ6438 as inducers of proliferation in organoids, as well as enhancing regeneration after epithelial damage *in vivo* [60]. However, organoids pose a challenge for investigators evaluating epithelial wound because of their closed nature (with the pseudolumen on the interior), which makes accessing the apical surface difficult and consistent wounding challenging. This model does still provide a means of studying important questions regarding the reparative process of intestinal epithelium in a physiologically relevant 3-D system. In addition, self-renewing monolayers, in which 3D culture is converted to 2D culture to create a monolayer, will likely be more amenable to studies of wound repair [61].

Cell lines

In addition to knowledge of the characteristics of epithelial wound models, an important component of any study is the epithelial cell type. There is significant utility in highly reductionist model systems that allow precise study of biological mechanisms. Although these models do not fully recapitulate the complexity of the intestinal mucosa, they do enable the selective study of epithelial wound repair, which is arguably the most vital component of the intestinal mucosal barrier [7, 21, 62]. Although transformed epithelial cell lines have been developed for convenient

usage in the lab, primary cell culture techniques have also undergone major advances to not only be more convenient for lab use, but also represent the multiple cell lineages that make up the epithelial barrier. While primary cultured epithelium more closely represents the *in vivo* environment, they have drawbacks related to passage time, confluence abilities, and susceptibility to experimental variation. These disadvantages can prevent investigators from understanding mechanisms of restitution, and therefore must be fully understood before embarking on further studies. In this section, some of the more commonly used cell lines that can be used for the study of restitution are described.

IEC-6

Quaroni *et al*. were one of the first labs to characterize an epithelial cell line that could be passaged multiple times when they developed IEC-6 cells[63]. IEC-6 cells are a non-transformed cell line derived from rat small intestinal crypt cells that mimic small intestinal epithelium *in vitro.* They are polygonal in shape with large nuclei and have microvilli present on one side of the cell demonstrating polarization [63]. They also express tight junction proteins, such as occludin, claudin-6, and ZO-1 as well as adherens junction proteins such as E-cadherin that are all crucial to adhere cells together into a uniform barrier [64]. In addition, their average peak TEER is ~30 $\Omega \cdot cm^2$ after 6 days of seeding [65], which mimics the 'leakier' TEER of small intestine. Originally, serial passages were made for ~6 months. From these passages, other researchers have been able to use these cells for various barrier function studies involving iron absorption [66], mitogen activated protein (MAP) kinases' role in apoptosis [67], and enteric protozoan's effect on TEER [65]. It must be noted that these cells mimic undifferentiated small intestinal crypt cells and therefore need additional coercion to differentiate. Fortunately, certain cell-medium additions such as transforming growth factor beta (TGF-β) [68], interferon gamma (IFN-γ), tumor necrosis factor

alpha (TNF-α) [69], and gastrin [70] have been found to successfully differentiate IEC-6 cells. Although differentiated IEC-6 cells seem to have only been used in one scratch wounding study investigating the role of polyamines and myosin II in cell motility [71],their undifferentiated counterparts have been widely used for different types of *in vitro* restitution studies. Most commonly, scratch wound models have utilized IEC-6 monolayers to investigate how various treatments like polyamine depletion[72], cytokine modulation[12], and Astragalus polysaccharides[73] can affect cell migration. These cells have also been used in *in vitro* hypoxia studies investigating how hypoxia affects barrier function [74] and different ways to protect against these hypoxic injuries [75, 76].

Caco-2

Researchers have found multiple tumor-based cell lines that have the ability to differentiate. One example is the Caco-2 cell line which was derived from a human colon adenocarcinoma and was developed in part due to the inability of researchers to passage and culture differentiated primary epithelial cells [9]. This transformed cell line spontaneously differentiates, and forms monolayers connected by tight junctions. These cells are absorptive, polarized, and have distinct apical, lateral, and basal cell membrane characteristics [9]. On their apical side they have microvilli similar to both enterocytes and colonocytes. [77] Further, Caco-2 cells do exhibit mature enterocytic properties such as disaccharidase and peptidase expression, more typical of small intestinal epithelium [78]. However, Caco- 2 cells are a transformed cell line, and have been shown to have gene mutations in p53, APC, β-catenin, and Smad4 which are uncharacteristic of intestinal epithelial cells [79]. Even with these mutations, Caco-2 cells are still widely used for intestinal studies focused on barrier functions such as barrier maintenance in the absence of

glutamine [80], tight junction modulation [81], and permeability response to short-chain fatty acids [82]. They have also been a part of multiple restitution studies involving varying model types. Caco-2 scratch assays have been performed to investigate how certain actions or factors enhance wound healing like the activation of protease activated receptor-2 [83]. Caco- 2 cells were also used to optimize the cell exclusion zone assay[46] as well as investigate how the loss of lipolysis-stimulated lipoprotein receptors affect barrier integrity [84]. In addition, these monolayers have undergone hypoxic injury to better understand the effect on barrier integrity. [85, 86].

There also exists a Caco-2 derived cell line, C2BBe1, that is more similar to the colon due to its brush border enzyme (BBe) expression. The BB of this cell line contains proteins such as villin, fimbrin, BB myosin I, fodrin, myosin II, and sucrase-isomaltase, all of which are found in the BB of colonocytes [87]. Given these unique properties, C2BBe1 has been used in a variety of intestinal studies. For example, C2BBe1 was used to study the effects of essential oils on inflammatory diseases [88] and recently used to investigate how SARS-Cov-2 affects the gastrointestinal tract [5]. However, C2BBe1 cells have not been used for restitution studies involving the models previously stated.

HT29

Another cell line originating from a human colon adenocarcinoma is HT29 cells. HT29 cells are more versatile than Caco-2 cells because they can accurately represent multiple cell lineages *in vitro*. In the presence of glucose concentrated media, these cells remain undifferentiated. When glucose is replaced by galactose, they differentiate into enterocyte-like cells [78]. In media highly concentrated with glucose and methotrexate (MTX), they begin producing large amounts of mucin,

mimicking goblet cells [77]. This led to the sub cell line called HT29-MTX. This cell line has been used to study drug transport [89] and protein digestion [90]. This cell line has also been used in conjunction with Caco-2 cells to study drug absorption [91] and toxicity [92]. In these co-culture studies, HT29-MTX cells are cultured with Caco-2 cells on a transwell at a biologically informed ratio of 1:9, respectively. In regards to wound studies, the HT29 cell line has been used to analyze how different biological components like MUC2 [93], beta-Adrenoreceptor antagonists[94], and protease activated receptor 2 agonists [95] influence restitution given a cell barren space either caused by scratch wound or cell exclusion zone assay. In addition, they have also been used to study epithelial integrity following hypoxic injury and the mechanisms whereby calcium channel blockade following this injury affects barrier integrity [96].

T84

With similar origins to Caco-2 and HT29 cells, T84 cells are also derived from human colon adenocarcinoma; however, these cells originated from a metastatic lung tumor. T84 cells possess similarities to Caco-2 cells such as their absorptive properties, spontaneous differentiation post confluence, and columnar cell physiology with apical microvilli [97]. These similarities have led researchers to use both cell lines interchangeably as a monolayer model of intestinal epithelial cells. However, some studies have shown that T84 cells act more like undifferentiated crypt cells and colonocytes. T84 cells, like many intestinal cell lines, also exhibit atypically high measures of barrier resistance (Table 1). Madara et al. indicated that the T84 cell Cl^- secretory response was more similar to crypt cells' secretory response than mature absorptive epithelial cells [98]. In 2017, Devriese *et al*. built on this argument by showing the difference between T84 models and Caco-2 models [97]. More specifically, this study showed that T84 cells had 3-fold shorter microvilli than Caco-2 cells, which is a characteristic of colon epithelial cells. In addition, the colonic marker,

monocarboxylate transporter 1 (MCT1), was upregulated in differentiated T84 cells, while enterocyte brush border enzymes were not [97]. Based on these findings, and the fact that Cl^- secretion plays a large role in the regulation of diarrhea and constipation, T84 cells have been widely used as models for electrogenic Cl^- secretion in the colon [99-103]. This cell line has also been used to model restitution following injury. Scratch assays have been conducted to see how factors such as galectins [104] or microbiota-derived butyrate[105] affect re-epithelization. In addition, T84 cells have been used in conjunction with neutrophils, or PMNs, to better understand their role following intestinal injury. One study coupled a scratch wound assay with a PMN migration assay, and found that PMN adhesion to epithelial cells post-migration enhances cell proliferation thereby aiding in wound healing. They believed that although transmigrating PMNs do cause additional damage to the tissue, once they adhere to epithelial cells, ICAM-1, a luminal epithelial surface receptor, is engaged, and this initiates Akt and β-catenin signaling, both of which are important signaling pathways for cell proliferation [106]. In addition, as previously mentioned, Nusrat *et al*. utilized T84 monolayers to model a wound caused by PMN infiltration. From this model, they found that wound closure methods vary in regards to wound size. Lamellipodia extensions are the main method of closure for larger wounds while smaller wounds tend to form and contract an actinomyosin cytoskeletal ring [107].

IPEC-J2

The IPEC-J2 cell line is a non-transformed line derived from jejunal crypts of a neonatal pig, developed in 1989 [108]. Historically, these cells have been used for a variety of functional studies due to their similarity to small intestinal epithelia. Specifically, IPEC-J2 cells contain several tight junctional proteins, are capable of secreting mucins (specifically Muc1 and Muc3), and cytokines such as TNF-α, and expresses a number of toll like receptors (TLR-1, -2, -3, -4, -5, -6, -8, -9, and

-10) [109, 110]. However, one of the largest limitations of the IPEC-J2 line grown conventionally in 5-10% Fetal Bovine Serum has been its unusually high barrier resistance, with TEER values averaging upwards of 1000 $\Omega^2 \times cm^2$ [111]. Previous studies performed by Zakrewski *et al*. showed a dramatic decrease in TEER values of IPEC-J2 cells when grown in 10% Pig Serum, with average TEER being 200-400 $\Omega \cdot cm^2$after 14 days [111]. Recently, studies in our laboratory evaluating the effects of media containing Wnt3a, R-spondin, Noggin (WRN) on IPEC-J2 cells were performed demonstrating decreased TEER values in IPEC-J2 cells grown in WRN-conditioned media. TEER values in these studies were $50.3 \pm 2.3\ \Omega \cdot cm^2$ over 12 days as opposed to IPEC-J2s grown in conventional media which exceeded 2000 $\Omega \cdot cm^2$[112]. This suggests potential for IPEC-J2 cells to be manipulated using media typically used for organoid culture, and may represent a more physiologically relevant model of barrier function in vitro in the future [112]. IPEC-J2 cells have also been used when studying restitution mechanisms. For example, one study utilized scratch wounded IPEC-J2 cell monolayers to observe restitution during tryptophan supplementation and its effects on the CaSR/Rac1/PLC-γ1 signaling pathway [113]. Additionally, IPEC-J2 cells have been employed to explore the positive effect of sodium butyrate and identify it as an agent capable of recovering intestinal tissues after assault or injury [114].

Conclusion

The *in vitro* models used to study gastrointestinal pathophysiology are an integral component of mechanistic research. For example, the HT-29 and IPEC-J2 cell lines have contributed heavily to research in restitution mechanisms by performing scratch wounding assays and cell exclusion models. Similarly, IEC-6 cells have been utilized in neutrophil exclusion studies. The T84 cell line has been utilized to better understand neutrophilic injury during the reparative process.

Caco-2 cells have also been invaluable in studies seeking to understand restitution through ECIS and its novel system for modeling injury through electrical pulses. However, the success of these models lies in the ability of researchers to properly choose the cell line and model best suited for their study.

Future work in optimizing these modeling systems to create increasingly relevant models of the *in vivo* environment is necessary to speed the pace toward clinical application. In addition, the field of *in vitro* modeling systems of intestinal epithelium is expanding rapidly and new systems which rely on primary cells derived from individual patients and grown in monolayers or 3-D organoids are becoming commonplace. These systems will inevitably require alternative or adapted methods of *in vitro* injury and repair to assess their viability properly and accurately as a model of restitution after insult.

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Table 1. Comparison of intestinal cell lines. Brief comparison of cell lines summarizing major characteristics of each.

Cell line	**Origin**	**Transformed (Y/N)**	**Average TEER**	Notes	**Reference**
IPEC-J2	Neonatal porcine jejunum	N	500-4000 (Ω X cm2)	-Not known to possess mutations -Differentiates into epithelial cell lineage without coercion	[97]
Caco-2	Human colorectal adenocarcinoma	Y	150-400 (Ω X cm2)	-Form distinctly polarized monolayer -Possess apical microvilli and are phenotypically similar to small intestinal epithelial cells -Known mutations	[104], [105]
T84	Human colon adenocarcinoma	Y	2,000-4,000 (Ω X cm2)	-Derived initially from lung -Used in study of colonic Cl- secretion	[106]
HT-29	Human colon adenocarcinoma	Y	500-600 (Ω X cm2)	-Can produce mucin, act as goblet cells -Differentiates into enterocytes in presence of glucose	[107, 108]
IEC-6	Rat small intestinal epithelium	N	42 > (Ω X cm2)	-Exhibit crypt-like phenotype, need media additives to differentiate	[109]

Table 2. Comparison of *in vitro* injury models. Advantages and disadvantages of each *in vitro* injury model.

Injury model	Advantages	Disadvantages	Notes
Scratch Assay	• Can be used on any cell line or primary culture able to form monolayers • Technically simple • Ability to adjust size and location of wound	• Inability to standardize each scratch, creating variability • Can disrupt ECM	• Concerns remain regarding the ability to differentiate between cell migration, restitution and proliferation. Edu labeling can be used to clarify results
Cell Exclusion Zone Assay	• Does not actively injure monolayer, therefore does not damage ECM • Uniform pattern, standardized	• Because it is a passively created hole, some do not consider this a 'wound'	• Only used to evaluate migratory or cell 'crawling' ability
Neutrophil wounding models	• Ability to evaluate secondary effects at the site of injury other than the physical wound • Able to manipulate chemoattractant levels as well as oxygen level	• Only evaluates wound formation and repair specific to neutrophil transmigration	• Method can be used to examine mechanisms of interepithelial junction repair
Electric cell-substrate impedance sensing	• Allows for direct observation of wound healing events • Allows of electrophysiological quantification of each phase of repair	• Heat from electrical current can cause additional damage • Density of monolayers can complicate measurement of impedance	• Opportunity to alter wound severity
Organoid models	• Enables study of the reparative process with crypt and villus domains • 3-D structure comparable to *in vivo* environment	• Difficult to access apical surface of epithelium • Can be difficult to assess repair due to the complex structure	• Is able to repair post- irradiation

Figure 1

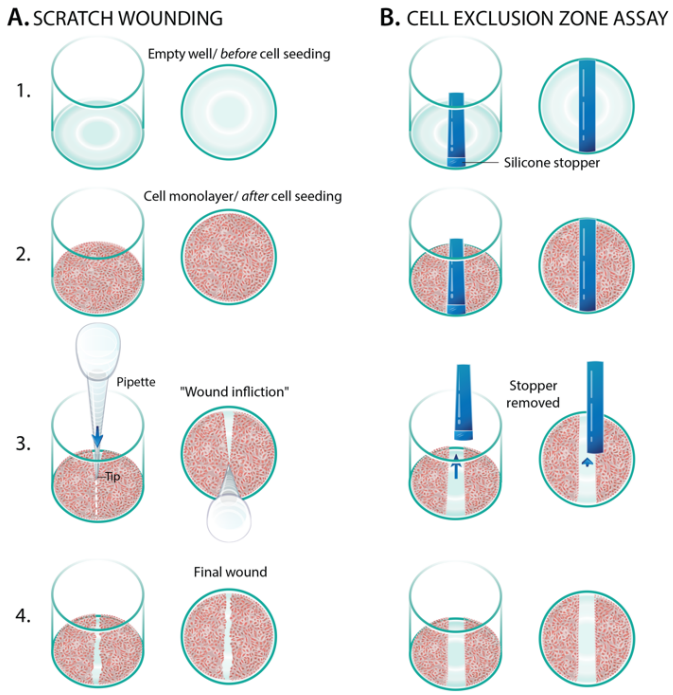

Figure 1. Key differences in wound structure between scratch wounding and cell exclusion zone assay methods. A) Scratch wounding with a pipette creates a natural and unsymmetrical wound. This can cause cellular damage during the scratch. B) Cell exclusion zone assay creates a wound with a clean-edge and minimal to no cellular damage as cells are grown around the stopper.

CHAPTER TWO

Establishment and Characterization of the Intestinal Porcine Jejunal Cell Line (IPEC-J2) Using Wnt-based Crypt Culture Media

Introduction

The development of functionally relevant translational models is critical to the study of gastrointestinal physiology. Recently, the use of porcine models of intestinal physiology have provided key insights to animal and human disease processes [1]. The porcine model is advantageous for translational studies due to the similarity of pigs to humans in size, nutritional requirements, immunological responses, and genome. For example, pigs are omnivorous and possess similar intestinal cellular lineages and architecture [2, 3]. Additionally, the porcine genome sequence homology of pigs to humans is 60%, while that of rodents is only 40% [2]. These characteristics contribute to the established success of pigs as a translational model of intestinal pathophysiology.

The comparison of most existing intestinal cell lines after continuous passaging to their corresponding *in vivo* environments has yet to be studied in depth. Many cell lines such as the Caco-2 cell line have been used extensively to model the small intestine, although these cells were originally derived from a human colon adenocarcinoma [4]. Similarly, T84 cells were derived from metastatic human colon carcinoma and have been used extensively to model the colon, but develop transepithelial electrical resistance (TEER) values far higher than normal colonic epithelium [5]. Transformed cell lines like Caco-2 and T84 also pose other concerns for *in vitro* translational studies. Specifically, the Caco-2 cell line has gene mutations in p53, APC, β-catenin, and Smad4 that are uncharacteristic of intestinal epithelial cells [6]. The T84 cell line has also been shown to contain mostly undifferentiated crypt cells and colonocytes [7]. Therefore, the use of non-transformed cell lines as a means of studying gastrointestinal disease and physiology may be advantageous.

The IPEC-J2 cell line was derived from porcine neonatal jejunum epithelium using primary culture techniques, but has been shown to be continuously passageable, most likely because of the inclusion of multiple cell lineages, including stem cells. The IPEC-J2 cell line retains the ability to form confluent monolayers, and forms a 'tight' barrier that includes the presence of many junctional proteins including claudins-1, -3, -4, -14, -16, occludin, and zonula occludens-1 (ZO-1) [8, 9]. In addition, this cell line has been shown to express TLRs -1, -2, -3, -4, -5, -6, -8, -9, -10 and IL-1α, -1β, -6, -7, -8, -12A, -12B, and -18 [10]. The presence of these physiological and immunological similarities to native tissue has led to the use of IPEC-J2 cells to study epithelial responses to important pathogens such as *Lawsonia intracellularis*, *Clostridium difficile* and *E. Coli* [11-13]. However, IPEC-J2 monolayers have shown atypically high measures of intestinal barrier function primarily composed of mature enterocytes while containing very few other differentiated cell types. The intestinal epithelial barrier is most permeable at the crypt base, where secretory responses are prominent, in part due to 'leakier' tight junctions with fewer junctional strands in the crypts [14, 15]. This 'leakiness' of the crypt base results in TEER values ranging from 46-59 $\Omega \bullet cm^2$ in neonatal tissues measured via Ussing Chambers [16]. In order to properly recapitulate this aspect of intestinal epithelial physiology, the relative quantity and lineage of cells typically found in the crypt base would optimally be present in *in vitro* modeling systems.

Three-dimensional primary cultured enteroids create a model of the intestinal structure composed of a basement membrane (Matrigel®) and an epithelial layer containing crypt-like structures enclosing a pseudolumen which closely mimics that of the *in vivo* environment [17]. The media developed to support this model contains three primary growth factors: epidermal growth factor (EGF), the Wnt agonist R-spondin, and Noggin (a bone morphogenic protein inhibitor) [18].

Additionally, Wnt-3a was eventually added in order to nourish the population of $Lgr5^+$ stem cells to effectively drive proliferation of epithelial cells in the pseudocrypt of the enteroid [19]. These factors have proven to drive a 3-D model of the intestinal epithelium by appropriately stimulating stem cells of the crypt base to proliferate and mature into differentiated cell types, including transit amplifying cells, enterocytes, enteroendocrine cells, and goblet cells as they migrate towards the apical surface of the enteroid epithelium. However, utilizing these models to observe effects on barrier function or to evaluate potential therapeutics has proven difficult as methods to use microinjection to insert contents into the pseudolumen of these enteroids is inconsistent and often ineffective.

To study permeability assays or mechanisms related to barrier function, 2-D monolayer systems are needed in order to observe migration of substances across the intestinal epithelium. One study seeking to investigate this in human intestinal epithelium developed self-renewing 2-D monolayers from human endoscopic biopsy and cultured them with media containing Wnt3a, R-spondin, and Noggin [20]. This study is just one example of using a human 2-D primary culture as a means for studying barrier-related monolayer properties with TEER values averaging 400 $\Omega \bullet cm^2$ [21]. Additionally, cells grown in this environment were capable of forming spheroids and displaying region-specific development of mature epithelial cell lineages. However, obtaining primary biopsies is financially costly as well as inconsistent as it relies on human donors. In order to study intestinal barrier function and physiology, a consistent, effective model is needed. Similarly, murine models have previously proven difficult to translate to humans and pose challenges including lack of clinical relevancy [22, 23]. For example, TEER of murine primary culture was recorded to be 500-2000$\Omega \bullet cm^2$, far exceeding that of the *in vivo* environment [21].

Taking into account previous models discussed, a more physiologically relevant, accessible, 2-D model of the intestinal epithelium for use in translational research is needed. In an effort to enhance the IPEC-J2 cell line as an *in vitro* model and overcome limitations associated with intestinal epithelial cell lines, the cell culture conditions utilized in 3-D culture were applied to IPEC-J2 cells in this study to encourage the presence of stem cell populations, increase cell heterogeneity, and more closely mimic barrier function of native tissue via introduction of Wnt, R-spondin, and Noggin to cell culture media.

Methods

Cell line and culture conditions

The IPEC-J2 cell line is a non-transformed cell line derived from porcine jejunal epithelia isolated from a neonatal pig less than 12-hours-old [24]. These cells were grown in standard DMEM media with 5% FBS, 1% Insulin/Selenium/Transferrin, 0.5% Epidermal Growth Factor, and 0.125% Penicillin/Streptomycin. Cells were maintained in a humidified 37°C, 5% CO_2 incubator. Media changes were performed every 24-48 hours. For IPEC-J2 cells grown in a Wnt, R-spondin, and Noggin enriched environment, media was composed using components found in **Table 1**. Incubation and media changes were the same as those listed for standard media.

Ussing chamber studies

Porcine tissues were harvested from <2-week-old neonatal piglets and 6- to 8-week-old juvenile piglets and immediately placed in oxygen-rich (95% O_2-/5% CO_2) Ringers' solution (mM: Na^+, 154; K^+, 6.3; Cl^-, 137; HCO_3^-, 24; pH 7.4). Tissues were then mounted in 0.67-cm^2 aperture

Ussing chambers. Mean data are representative of Ussing chamber experiments (n=5-7 for neonatal tissues) (n=14 for juvenile). The spontaneous potential difference (PD) was measured using Ringer-agar bridges connected to calomel electrodes, and the PD was short-circuited through Ag-AgCl electrodes with a voltage clamp that corrected for fluid resistance. TEER ($\Omega \bullet cm^2$) was calculated from the spontaneous PD and short circuit current (Isc). If the spontaneous PD was between -1.0 and 1.0 mV, tissue was current clamped at 100A for 5s and the PD recorded. After tissues were mounted on Ussing chambers, they were allowed to acclimate for a period of 30 min. Isc and PD were recorded every 15 minutes over a 120-minute experiment.

Isotopic Mannitol flux studies

All fluxes were conducted under short-circuit conditions (tissue clamped to 0 mV). ^{3}H-mannitol (0.2 µCi/ml diluted in 10mM mannitol) was placed on the mucosal side of tissues. During *ex vivo* experiments, two 60-minute fluxes from 0- to 60-minutes and from 60- to 120-minutes of the experimental recovery period by taking samples from the opposite side of that of isotope addition and counted for ^{3}H ß-emission in a scintillation counter. Mucosal-to-serosal fluxes (J_{ms}) of mannitol were calculated using standard equations.

Transepithelial electrical resistance (TEER) measurements on cell monolayers

Cells were passaged a minimum of five times and seeded at a density of 1 x 10^4 cells/mL on 0.4 µm pore-sized Snapwell filters (polycarbonate or clear polyester) (Corning, The Netherlands) coated with rat tail collagen 1 (Serva, Germany). Transepithelial electrical resistance (TEER) measurements were performed using a Chopstick Electrode Set (WPI, Sarasota, FL, USA)

positioned on the apical and basal sides of the monolayers and attached to an Epithelial Volt Ohm Meter 2 (WPI). For all TEER measurements, the inserts were plated at an equal density; the readings were taken in triplicate per monolayer and averaged. The TEER recorded from blank inserts with no cells was subtracted from the TEER of inserts with cells.

RT-qPCR

Mucosal scrapes were obtained from neonatal and juvenile porcine jejunum using a glass slide to separate the mucosa and submucosa from the muscle and serosal layers and then flash frozen in liquid nitrogen and stored at -80°C. Total RNA was isolated from cells with the RNeasy Mini kit (Qiagen, Valencia, CA), treated with the Qiagen RNase-free DNase Set, and quantified spectrophotometrically to determine the quality and yield (NanoDrop Technologies Thermo Fisher Scientific Wilmington, DE). After quantification, 2ug of RNA was converted to cDNA using iScript cDNA synthesis kit (Biorad). Biorad thermocycler settings were set to 5 min at 25°C, cDNA synthesis at 42°C for 30 min, denaturation at 85°C for 5 min, and held at 4°C before being stored at -20°C. cDNA was diluted 1:4 and qPCR was performed with iTaq Universal SYBR Green Supermix (Bio-Rad Laboratories), the ABI QuantStudio 6 system (Applied Biosciences, Carlsbad, CA), and PCR primers obtained from Integrated DNA Technologies (Coralville, IA) for identification of *Villin, β-catenin, E-cadherin, Sox9, CgA, Muc2, HopX, PCNA, Ki67, SGLT1, Lgr5,* and *Olfm4*. Cycle parameters included polymerase activation and DNA denaturation at 95°C for 30 sec. Forty cycles of amplification were performed with a 15 sec denaturation at 95°C and annealing/extension for 60 sec at 60°C. The melting curve analysis was performed at 65°C–95°C with 0.5°C increments, 5 sec per step. Melting curves were checked to ensure consistent amplification of a single PCR product and

non-template controls were included to ensure there was no contamination. The data set was analyzed using the ΔΔCt method with β-actin as the reference gene.

Immunofluorescence analyses

IPEC-J2 cells were seeded at a density of 1 x 10^4 cells/mL on chambered slides and were washed thoroughly with PBS and fixed with cold (4°C) 4% paraformaldehyde (PFA) before being stored at −80°C until staining. The cells were thawed, rinsed in PBS, blocked overnight with protein block serum-free (DakoCytomation, Via Real Carpinteria, CA, USA), and incubated overnight at 4°C with primary antibodies in antibody diluent (DakoCytomation). The cells were washed thoroughly with PBS and incubated in secondary antibodies conjugated with fluorescent dyes, and nuclei were stained with DAPI (ThermoFisher Scientific, Waltham, MA, USA). Following rinsing in PBS, the cells were mounted on fluorescent mounting media (DakoCytomation) and examined with an Olympus IX83 Inverted Motorized Microscope with CellSens software (Olympus, Tokyo, Japan). The objective lenses used were ×10, ×20, and ×40 with numerical apertures of 0.3, 0.45, and 0.6, respectively.

Neonatal porcine tissues were fixed in 4% PFA for 20-24 hrs. at 4°C. Tissues were then transferred to a 30% sucrose solution for at least 48hrs at 4°C before being embedding in OCT (optimal cutting temperature) media and frozen at -80°C. The tissues in OCT were then sectioned at 5-7 mm thickness using a cryostat and mounted on positively charged glass slides and stored at -80°C. For immunofluorescence, the tissues were removed from -80°C and washed thoroughly with PBS to remove OCT. When required, heat induced antigen retrieval was performed by placing slides in reveal decloaker solution for 30 seconds at 120°C and then 10 seconds at 90°C in a pressure cooker. The slides were then cooled and permeabilized in 0.3%

Triton -100X-PBS for 20 minutes, washed thoroughly with PBS and coated in protein block serum-free (DakoCytomation, Via Real Carpinteria, CA, USA). Slides were then kept at 4°C overnight. Primary antibodies were then applied according to **Table 2** and incubated overnight at 4°C**.** Tissues were then removed and rinsed with PBS three times before applying secondary antibodies diluted at 1:500 in PBS according to **Table 2** for 45 minutes. Slides were counter stained with DAPI diluted at 1:500 and mounted using fluorescent mounting media (DakoCytomation).

Larazotide Acetate

Pharmacology of Larazotide Acetate was observed using confluent IPEC-J2/WRN monolayers grown on 0.4 μm pore-sized Snapwell filters (polycarbonate or clear polyester; Corning, The Netherlands) coated with rat tail collagen 1 (Serva, Germany). After monolayers became confluent, 10mM larazotide acetate was apically applied to the IPEC-J2/ WRN cells and TEER measured using EVOM probes over an 8-hour time course.

3-D Enteroid-like Culture

To encourage the formation of 3-D enteroid-like structures, 50% media/ 50% Matrigel® patties were pipetted onto 6 well polystyrene plates using either standard or WRN media. Cells within the domes were seeded at a density of 1 x 10^4 cells/mL and allowed to grow for 7 days with regular media changes every 24-48hrs.

Statistical analysis

Data is reported as means ± SE. Differences between groups were tested with repeated measures two-way ANOVA with Bonferroni's multiple comparison tests (GraphPad Prism 9). Where appropriate, the difference between two groups were assessed with a student's *t*-test. $P < 0.05$ was considered significant for all statistical analysis.

Ethics Statement

All animal studies were approved by the Institutional Animal Care and use Committee at North Carolina State University.

Results

IPEC-J2 cells grown in WRN media exhibit TEER values similar to that of ex vivo native tissues

In order to evaluate the effects of Wnt-3a, R-spondin, and Noggin on the barrier function of IPEC-J2 cells, cell monolayers were cultured using conventional media and WRN-supplemented media. EVOM probes were employed to measure TEER of IPEC-J2 cells after five passages in culture over a 9-day period, revealing lower TEER (47.1 ± 1.8 Ω•cm^2) in IPEC-J2/ WRN cultured cells as compared to IPEC-J2 cells grown routinely in standard cell culture media (1,856 ± 109.2 Ω•cm^2, P<0.05). The 'leaky' epithelial barrier function of the WRN cultured IPEC-J2 cells was similar to that of neonatal (47.8 ± 6.9 Ω•cm^2) and juvenile (41.0 ± 2.6 Ω•cm^2) porcine tissues-maintained *ex vivo* in Ussing chambers (Fig. 1). Both neonatal and juvenile tissues had similar levels of mannitol flux on the Ussing chambers, with no significant change over two hours in either group (Figure 1).

Neonatal porcine epithelium displays heterogeneity in cell lineages, with increased expression of stem cell markers compared to juvenile tissues

To understand the relative proportions of cell lineages present *in vivo*, gene expression in neonatal (< 2-weeks-old) and juvenile (6-8-weeks-old) pigs was studied using RT-qPCR. This was also performed in order to highlight specific changes present in neonatal tissues as IPEC-J2 cells were originally derived from this age of pig. The RT-qPCR results confirmed a significant increase ($P<0.05$) in *Sox9, CgA, Sglt1, Lgr5,* and *Olfm4* in neonatal tissues compared to juvenile tissues (Figure 2). The stem cell lineages associated with *Sox9*, *Lgr5*, and *Olfm4* highlight an increase in proliferative cells found in the crypt base of native tissues.

Evidence of tight junction and epithelial proteins found in IPEC-J2 cells

To further investigate permeability changes observed between the two IPEC-J2 groups, we utilized immunofluorescence to identify known interepithelial junctional proteins. Adherens junction proteins, including, β-catenin, E-cadherin, and the epithelial marker villin were visualized in neonatal tissues and were also present in IPEC-J2 cells grown in both medias (Figure 3). The tight junction protein occludin was also present in IPEC-J2 cells (Figure 3b).

IPEC-J2 cells grown in media conditioned with Wnt, R-spondin, and Noggin display greater heterogeneity in cell lineages

Bright field microscopy was initially used to visualize morphology of the cells grown in IPEC-J2 conventional and WRN media. Cells grown in WRN media appeared to display greater heterogeneity and diversity of cell types. Specifically, what looked like secretory elements were observed in IPEC-J2/WRN (Figure 4).

Using immunofluorescence, neonatal tissues were evaluated for the presence of both stem cell and differentiated cell lineages, confirming the presence of Sox-9, CgA, and Muc-2 (Figure 5). Additionally, immunofluorescence revealed the presence of Sox-9, CgA, and Muc-2 in both IPEC-J2 conventional and IPEC-J2/WRN cultured cells, as well as other proliferative markers (Hop-X, PCNA, and Ki67) (Figure 5b). To compare the relative quantities of various cell lineages across IPEC-J2 cells grown in conventional versus WRN media, cell counting was performed using ImageJ software to select positive cells across images. The quantified imaging results indicated a significant increase ($P<0.05$) in Sox-9^+, Hop-X^+, Ki67^+, Muc-2^+, and CgA^+ cells in the IPEC-J2/WRN group when compared to IPEC-J2 cells grown in standard media (Figure 6).

IPEC-J2 cells as a 3-D model of intestinal epithelial cells

IPEC-J2 cells have been established as a sustainable model of intestinal epithelium in 2-D modeling systems. However, we sought to identify if IPEC-J2 cells could be transformed into a 3-D *in vitro* model of the epithelium. In order to evaluate 3-D formation, Matrigel was incorporated into both WRN and standard media and cells seeded at $1x10^4$ cells/well. Over 5 days, the presence of enteroid-like structures appeared visibly in IPEC-J2/WRN when viewed through an inverted light microscope (Figure 7). The IPEC-J2/WRN enteroid appeared to contain advanced organization whereas IPEC-J2 cells grown in standard media did not seem to truly form visible 3-D structures.

IPEC-J2 cells as a pharmacologic screening model for assessing barrier function after treatment with Larazotide Acetate

To assess whether or not IPEC-J2 cells may have utility in pharmaco-physiological studies, IPEC-J2 cells in WRN media were studied to determine the effect of the novel tight junction regulator, larazotide acetate. This peptide has been shown to regulate barrier function of 'leaky' epithelia via a selective action on tight junction proteins [28, 29]. IPEC-J2/WRN cells treated apically with 10mM larazotide display significantly ($p<0.01$) increased TEER (Figure 8). Specifically, larazotide treated cells attained 58.8 ± 0.9 Ω•cm^2 at 8 hours as compared to untreated cells (52.6 ± 1.33 Ω•cm^2) at 8 hours. This indicates that larazotide acetate was able to increase intestinal barrier function in the 'leaky' intestinal monolayer system and demonstrates the IPEC-J2/ WRN system has the potential to be used as a pharmaceutical screening model.

Discussion

This study successfully established a novel method of culture for an intestinal epithelial cell line which displays physiologically relevant measures of barrier function and appropriate proportions of critical cell lineages found *in vivo*. Developing sustainable *in vitro* models for the study of gastrointestinal physiology and disease is critical for the advancement of translational research. The use of intestinal epithelial cell lines has the potential to provide an efficient, consistent, and accessible tool for such studies. The ability of cell lines to maintain relevant measures of barrier function that reflect that of native tissues has been difficult to achieve. Previous work in IPEC-J2 cells grown in standard media found TEER measurements as high as 2,000 Ω·cm^2 [9]. However, in IPEC-J2/WRN cells, TEER values mirror those of native neonatal and juvenile porcine tissues (47.1 ± 1.8 Ω•cm^2) (Figure 1). The changes observed in TEER data between the two media in

this study suggest cells grown in WRN media exhibit differences in tight junctional organization within cells associated with the stem cell niche, resulting in a 'leakier' epithelial barrier. Qualitatively, there were differences in the appearance of occludin, a tight junction protein, as well as differences in the appearance of adherens junction proteins E-cadherin and β-catenin between IPEC-J2 cells grown in differing media. However, further studies would be required to assess membrane localization and quantification to fully understand the relationship of junctional structures to TEER in the current study.

In addition to the presence of interepithelial junctional proteins evident in immunofluorescence imaging, the presence of stem and proliferative cell types typically found in the crypt base may serve a crucial role in both barrier function and organization of the monolayer. Paracellular permeability is known to be higher in the crypt base and lower in the villi in the small intestine due to the relative proportions of tight junction strands present [15, 25]. The increased presence of $Sox9^{+}$, $HopX^{+}$, and $Ki67^{+}$ cells in IPEC-J2/WRN culture would therefore indicate a more crypt-like cellular environment in the IPEC-J2/WRN system, and the crypt is known to be more 'leaky' in native tissues. Interestingly, RT-qPCR revealed that *Sox9, HopX, Lgr5, Olfm4,* and *Ki67* were all significantly increased in neonatal tissues as compared to juvenile tissues (Figure 2), also suggesting that a more crypt-like IPEC-J2/WRN cell line is more physiological. Additionally, this increase in markers of progenitor/proliferative populations alongside differentiated cell types such as Muc2 (a marker of goblet cells) and CgA (a marker of enteroendocrine cells), mirrors the cellular heterogeneity exhibited *in vivo*.

Previous studies have investigated the idea of media supplementation to produce a reduction in barrier resistance of IPEC-J2 cell monolayers and allow for further study of tight junction protein physiology. Specifically, a study by Zakrewski showed evidence that supplementation of IPEC-

J2 cells with pig serum instead of fetal bovine serum resulted in a significant reduction in barrier resistance (TEER) in IPEC-J2 monolayers [26]. This change was apparently due to pig serum possessing a substance whose secretion by IPEC-J2 had been lost over the years as the cells have been extensively passaged [26]. However, this study did not assess cell lineage changes that might explain a change in barrier function, as compared to the present study, which correlated a reduction in TEER with increased progenitor cell lineages in the presence of WRN.

Another exciting property of the WRN/ IPEC-J2 model shown in this study is the ability of this 2-D monolayer to develop a 3-D enteroid structure in the presence of Matrigel® (Figure 7). Enteroids grown in the IPEC-J2/WRN group appeared to exhibit a complex, "budded" structure, whereas IPEC-J2 cells grown in conventional media did not produce any identifiable 3D structures. This advancement provides the opportunity for future studies to explore the use of cheaper, accessible, and easily manipulated cell lines such as IPEC-J2 as a means for studying gastrointestinal physiology in a closed 3-D system.

To assess the potential translational use of the WRN-IPEC J2 model, we chose to evaluate a novel molecule currently under investigation for celiac disease (larazotide acetate, LA) for its ability to change TEER of leaky epithelia. LA is a novel tight-junction regulator drug in phase III clinical trials for use in individuals with celiac disease [27]. LA was developed with the intent to restore the integrity of the epithelial barrier and decrease paracellular permeability to prevent the passage of gliadin through the paracellular space [28]. Using the IPEC-J2/WRN monolayer as a model for 'leaky' epithelium allowed for the *in vitro* observation of the effects of LA as a regulator of intestinal permeability. The increase in TEER of the monolayer measured as a response to apical application of LA demonstrates a potential use of IPEC-J2/WRN as a model for pharmacological screening when evaluating properties related to barrier function (Figure 8).

The present study has revealed the unique advantages of using the IPEC-J2 cell line as a relevant *in vitro* model for studying intestinal physiology and pathophysiology when cultured in media containing Wnt-3a, R-spondin, and Noggin to create *in vivo*-like conditions. Specifically, this model has displayed appropriate barrier function and heterogeneity of cell lineages previously unattainable in many porcine and murine cell lines. Additionally, stem and progenitor cell types commonly found in the crypt base and abundant in neonatal tissues were found to be present in the IPEC-J2/WRN *in vitro* system. Finally, this study revealed the potential use of IPEC-J2 cells as a 3-D modeling system, and the utility of IPEC-J2/ WRN as a model for pharmacological screening.

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Table 1. **IPEC-J2 cell line media composition.**

Original	**WRN**
DMEM/F12-50/50	50% L-WRN **(Wnt-3a, R-Spondin, Noggin)**
FBS	Advanced DMEM/F12
Pen/Strep	1XB-27
Ins/Sel/Trans	1XN2
EGF	2mM Glutamax
	10mM HEPES
	Pen/Strep
	N-Acetyl-L-cysteine
	EGF(1:3)

Table 2. Functional antibodies used in immunofluorescence assay.

Target	Maker	Cat. #	Source	1'Ab	2'Ab	Storage	Antigen Retrival
Villin	Invitrogen	PA5-22072	Rabbit	1:500	Cy3-anti-rab	4C	Y
β-catenin	Cell signaling	2677	Mouse	1:1000	Al488-anti-ms	-20C	N
E-cadherin	Invitrogen	PA5-9527	Rabbit	1:1000	Cy3-anti-rab	-20C	N
Occludin	Invitrogen	33-1500	Mouse	1:100	Al488-anti-ms	-20C	N
Sox9	Millipore	Ab5535	Rabbit	1:1000	Al568-anti-ms	4C	Y
CgA	Immunostar	20086	Mouse	1:500	Al568-anti-ms	-20C	N
Muc-2	Invitrogen	MA5-12345	Mouse	1:100	Al568-anti-ms	4C	N
Hop X	Santa Cruz	Sc-30216	Rabbit	1:500	Cy3-anti rab	4C	Y

Figure Legend

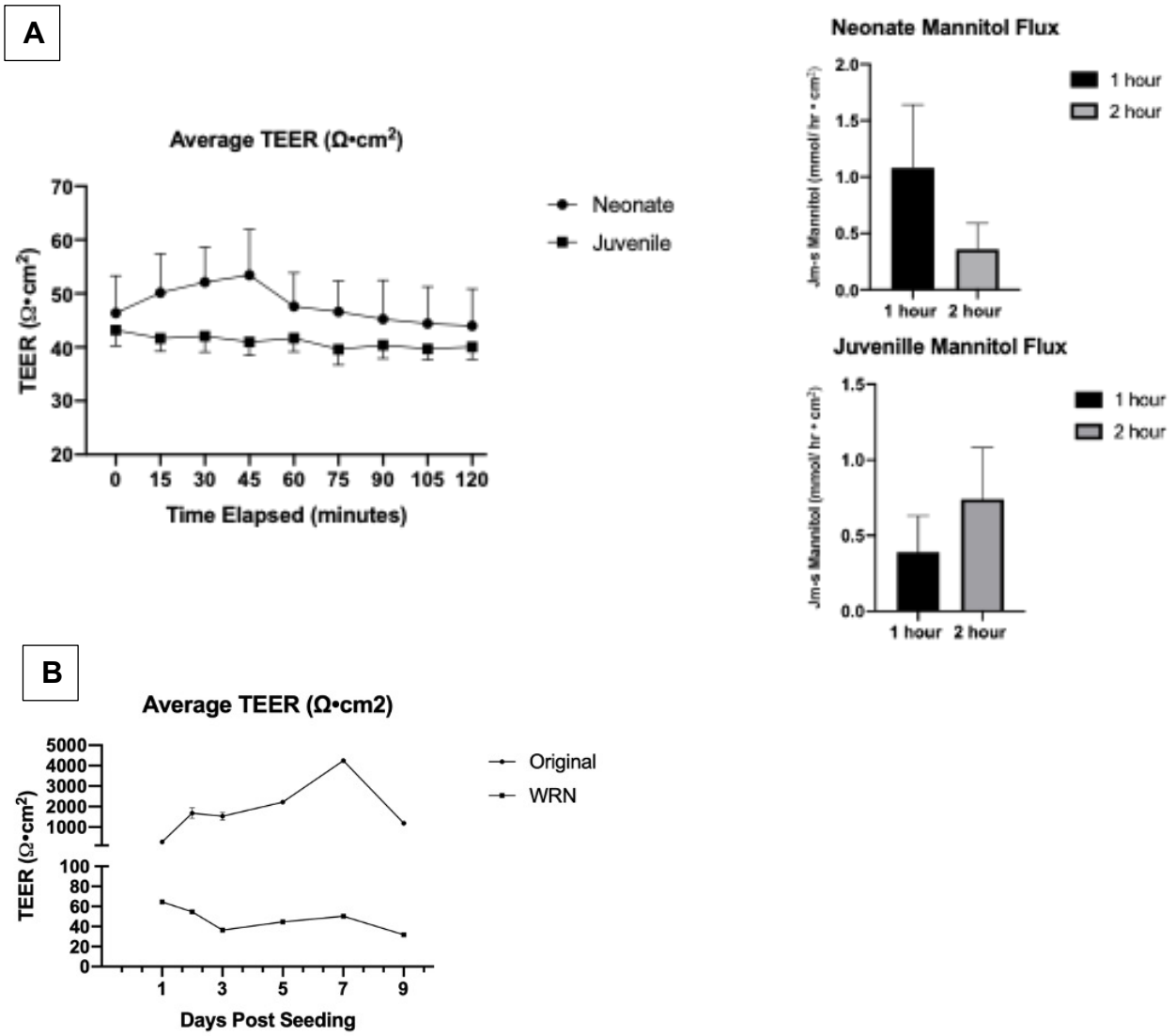


Figure 1. IPEC-J2 cells grown in WRN media exhibit TEER values similar to that of ex vivo native tissues. A) TEER over 120-minutes ex vivo in Ussing chambers. Average TEER in neonatal tissues (47.8 ± 6.9 Ω•cm2) was similar to that of juvenile (41.0 ± 2.6 Ω•cm^2) (n=5-7 for neonatal tissues) (n=14 for juvenile). B) Mannitol flux (Jm-s Mannitol mmol/hrcm2) run over two hours in neonate and juvenile tissues ex vivo using Ussing chambers. Note no change between neonate and juvenile groups. C) TEER in IPEC-J2 cells grown in standard (original) media and WRN media measured over nine days post-seeding. IPEC-J2/ WRN cultured

cells produced lower TEER (47.1 ± 1.8 Ω•cm^2) than IPEC-J2 cells grown routinely in standard cell culture media (1,856 ± 109.2 Ω•cm^2, P<0.05).

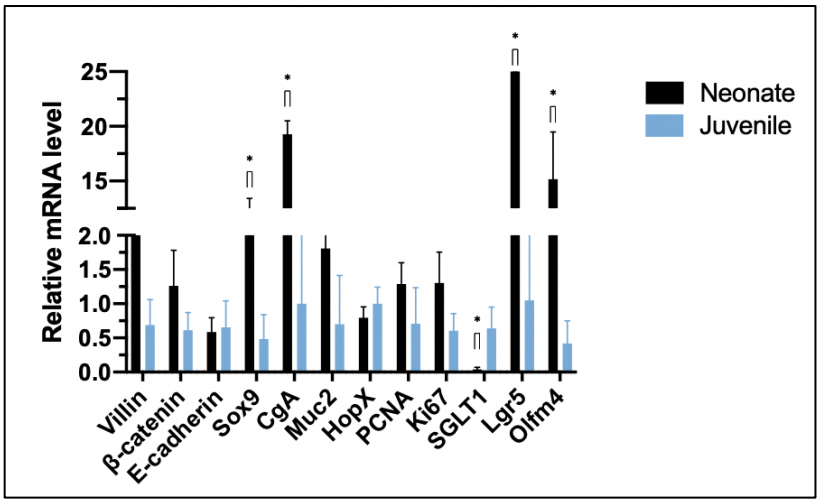


Figure 2. Neonatal porcine epithelium displays heterogeneity in cell lineages, with increased expression of stem cell markers compared to juvenile tissues. mRNA levels for *Villin, E-cadherin, B-catenin, Sox9, CgA. Muc2, HopX, PCNA, Ki67, SGLT1, Lgr5*, and *Olfm4* in neonate and juvenile tissue. Neonatal tissues exhibit significantly higher (P<0.05) of *Sox9, CgA, Sglt1, Lgr5*, and *Olfm4.*

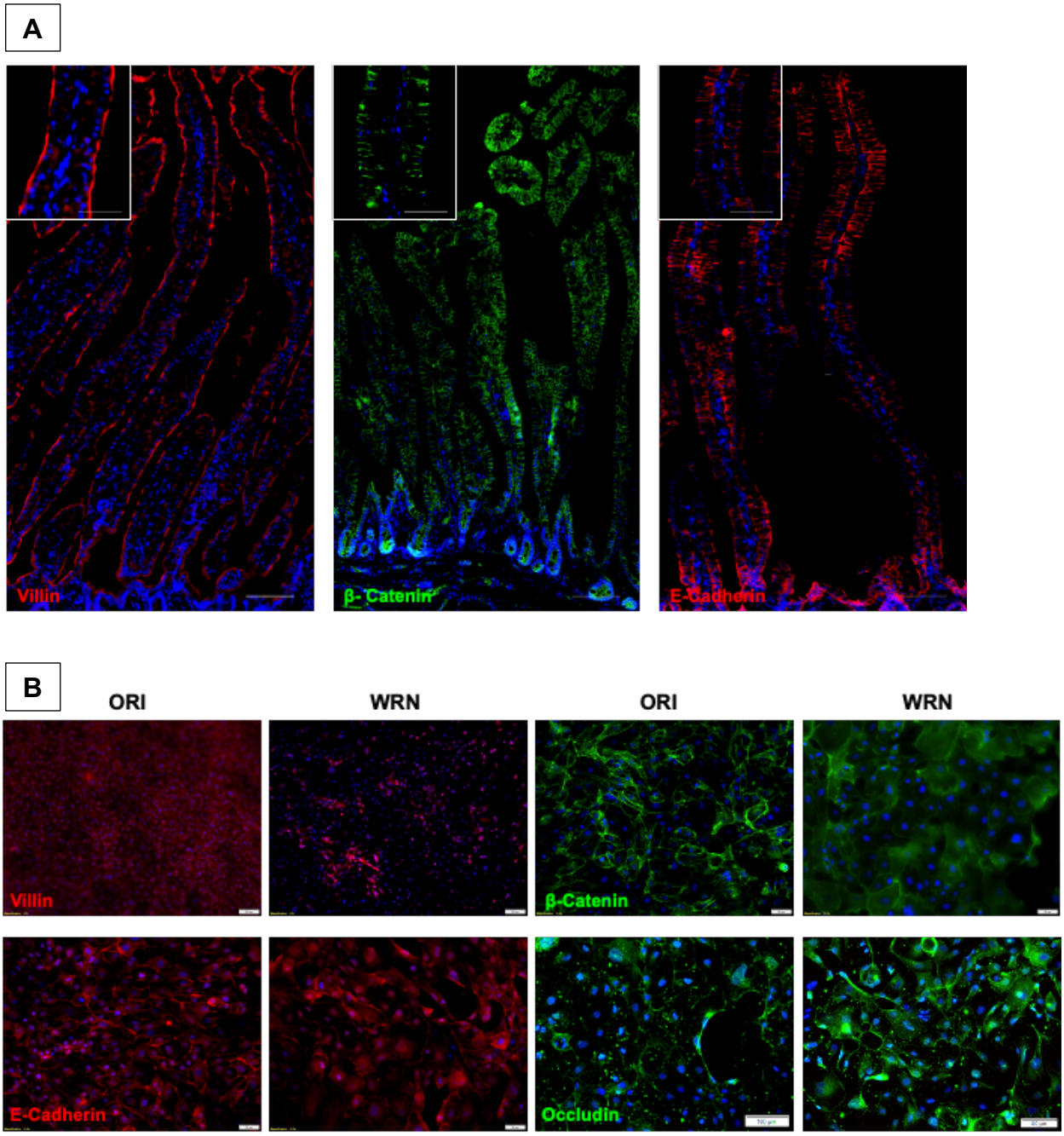


Figure 3. Evidence of tight junction and epithelial proteins found in neonatal tissues are also present in IPEC-J2 cells. A) Immunostaining for β-catenin, E-cadherin, known proteins associated with tight junction organization and function are located at cell junctions and on apical surface of the cell in neonatal small intestine. Villin, a protein associated with cytoskeletal structural integrity and microvillar actin filaments is also present on the apical surface. Scale bar set at 100µm. B) Immunostaining for β-catenin, E-cadherin, Villin, and Occludin in IPEC-J2

cells grown in standard media and IPEC-J2/WRN. Scale bar set at 100µm for Villin and Occludin, 50mm for E-Cadherin and B-Catenin.

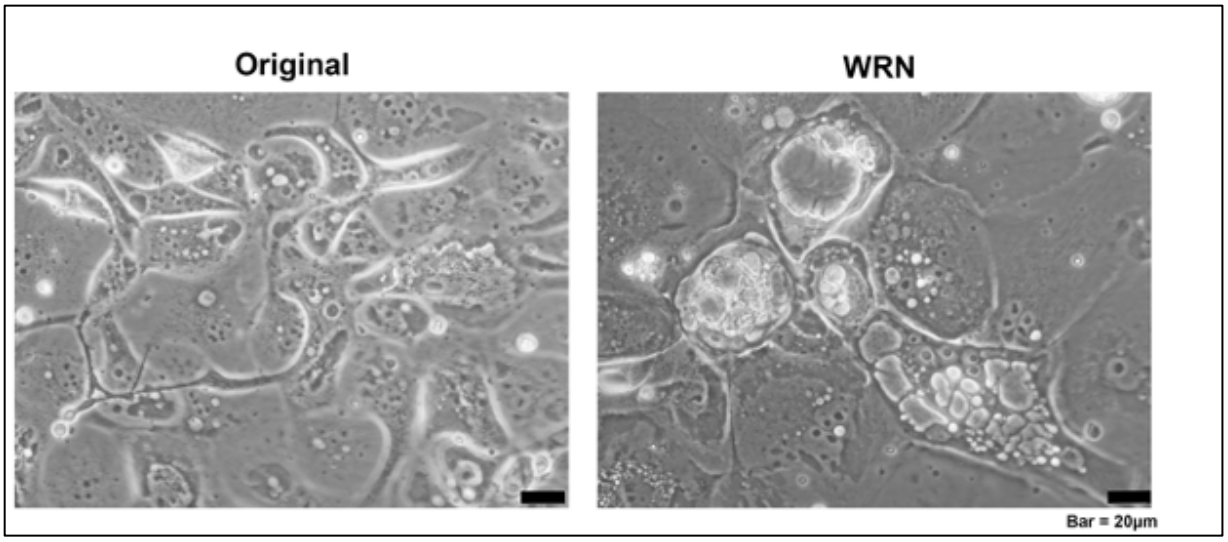
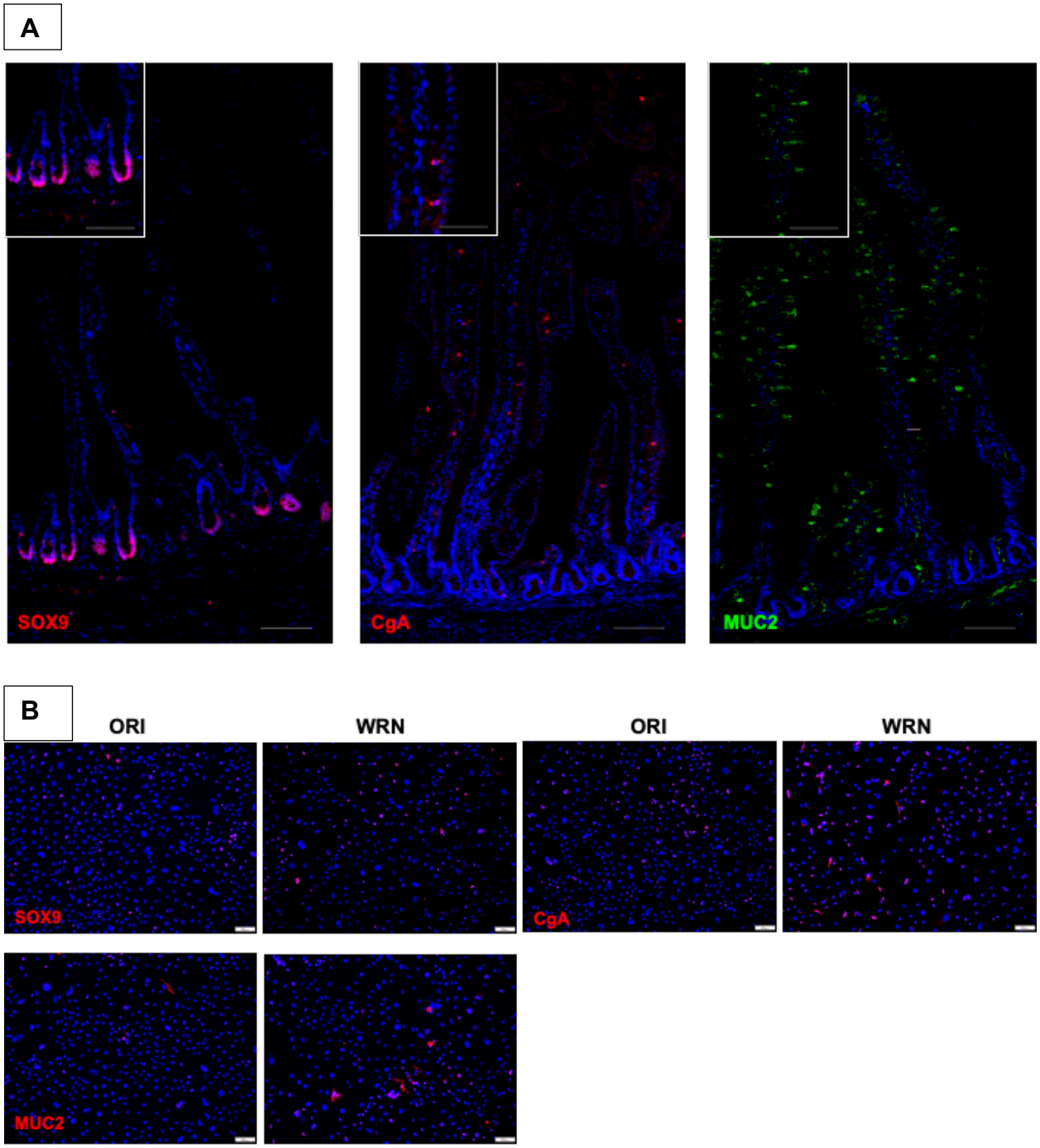


Figure 4. Cell morphology of IPEC-J2 cells in conventional and media supplemented with Wnt, R-spondin, and Noggin. IPEC-J2 cells grown in WRN media display complex elements including the appearance of secretory vesicles. Scale bar set to 20µm.

Figure 5. IPEC-J2 cells grown in media conditioned with Wnt, R-spondin, and Noggin display greater heterogeneity in cell lineages. A) Immunostaining for SOX9, associated with proliferative crypt-base columnar cells, CgA, and enteroendocrine secretory marker, and MUC2 an indicator of goblet cells, all identified along the epithelial axis in neonatal small intestine. Scale bar set to 100µm. B) Immunostaining for SOX99, CgA, MUC2, HOPX, PCNA, and KI67 in IPEC-J2 cells grown in standard media and IPEC-J2/WRN. Scale bar set to 100µm.


A
SOX9
CgA
MUC2
B
ORI
WRN
ORI
WRN
SOX9
CgA
MUC2

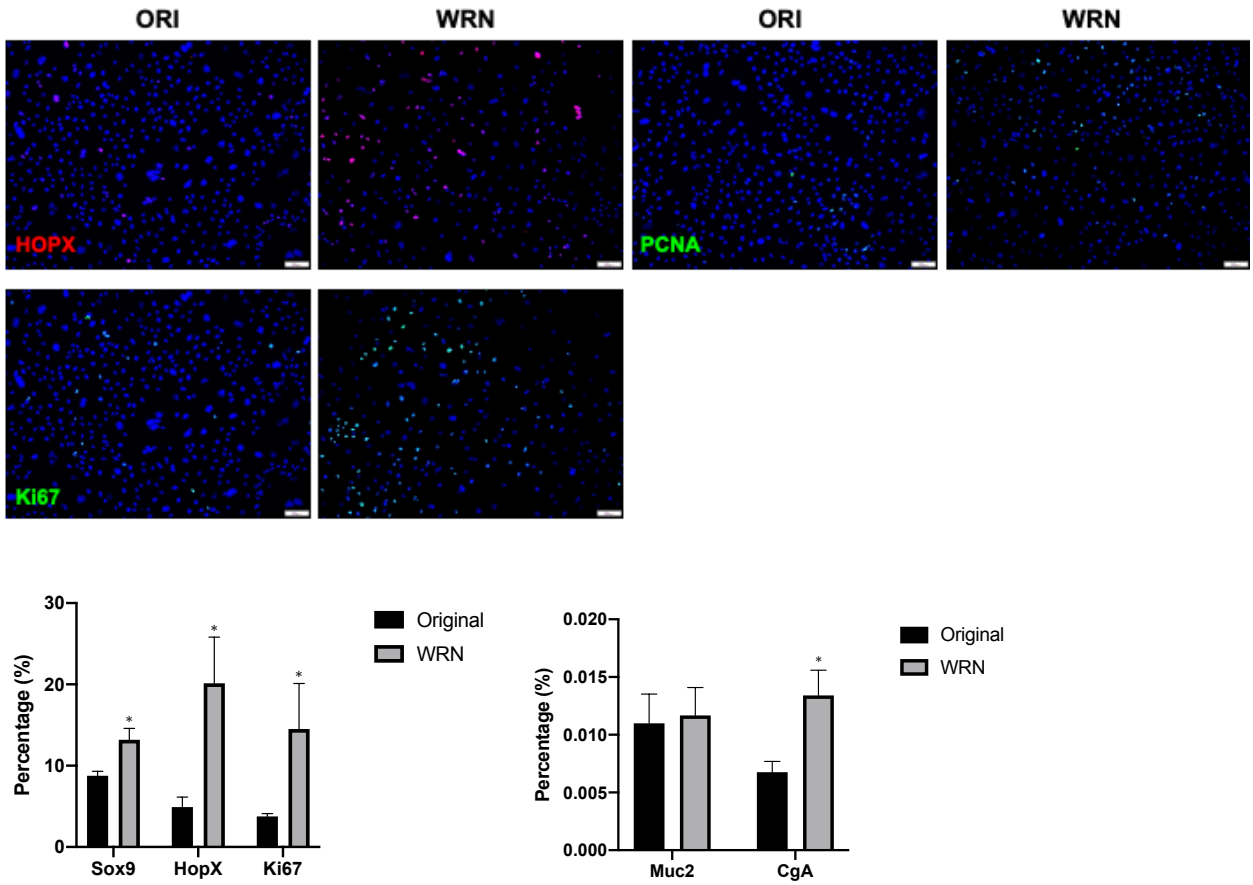


Figure 6. IPEC-J2 cells grown in WRN media displayed increased heterogeneity.

Immunostaining analysis of IPEC-J2/WRN showed a significant increase in expression of Sox-9^{+}, Hop-X^{+}, Ki67^{+}, Muc-2^{+}, and CgA^{+} cells via two-way ANOVA, P≤0.001; **P≤0.01; ***P≤0.001 by multiple comparisons test. Analysis was performed using ImageJ software to select positive cells across images at the same exposure.

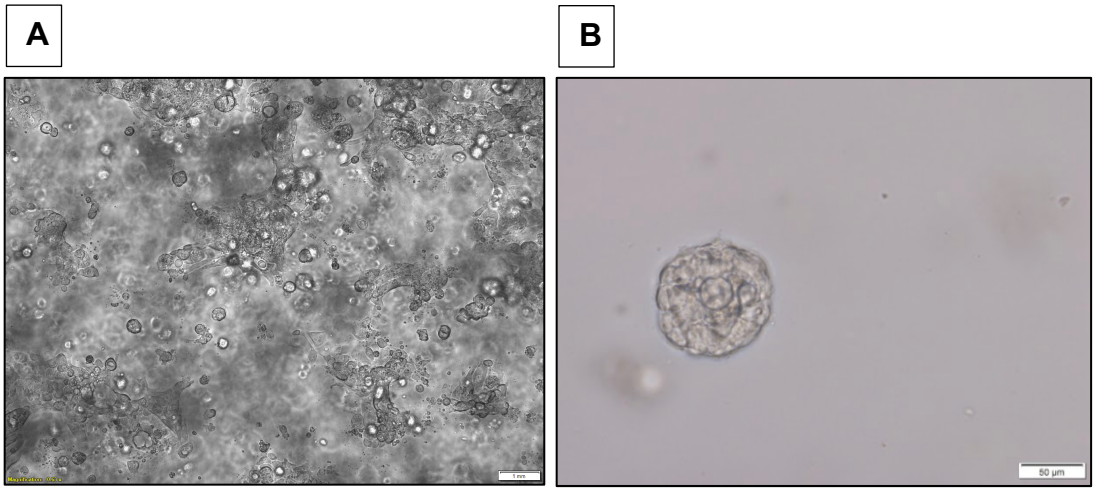


Figure 7. IPEC-J2 cells as a 3-D model of intestinal epithelial cells. A) IPEC-J2 cells grown in standard media after 3 days in post-seeding. B) IPEC-J2/WRN cells five days post-seeding when suspended in Matrigel. Structures were more complex and visible as opposed to IPEC-J2 grown in standard media.

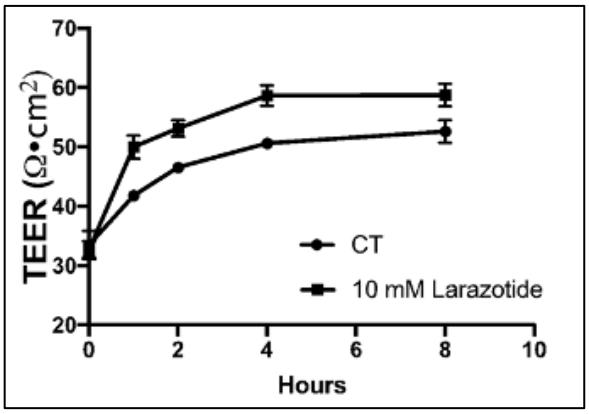


Figure 8. IPEC-J2 cells as a pharmacologic screening model for assessing barrier function after treatment with Larazotide Acetate. Average TEER over 8 hours in IPEC-J2/WRN monolayers treated apically with 10 mM Larazotide Acetate. Larazotide acetate produced a significant increase ($P<0.01$) in TEER over 8 hours with treated cells reaching 58.8 ± 0.9 Ω•cm^2 and untreated cells measuring 52.6 ± 1.33 Ω•cm^2.