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Los Angeles

The Study of Intestinal Epithelial Monolayer Development

and

Its Interaction with Intestinal Subepithelial Myofibroblast

A dissertation submitted in partial satisfaction of the

requirements for the degree Doctor of Philosophy

in Bioengineering

by

Po-Yu Lin

2019

ABSTRACT OF THE DISSERTATION

The Study of Intestinal Epithelial Monolayer Development

and

Its Interaction with Intestinal Subepithelial Myofibroblast

by

Po-Yu Lin

Doctor of Philosophy in Bioengineering

University of California, Los Angeles, 2019

Professor James C. Y. Dunn, Chair

The goal of biomedical research is first to understand the mechanism of the diseases, then develop a therapy to mitigate such disease. In the process, animal models and in vitro culture systems comprising primary human cells would be utilized as a study platform. An in vitro system representing small intestine is of great interest. However, attempts to develop an in vitro system which accurately reflect the phenotype and function of the human small intestinal epithelium has so far proven unsatisfactory. Hence the accuracy of the experimental results conducted on such systems might be hampered. The goal of this research is to develop an in vitro intestinal epithelium model which accurately recapitulate the human epithelium in vivo.

A natural progression of the epithelial monolayer development was observed in vitro. The morphology of the monolayer went from organized toward disorganized as the cells underwent apoptosis. The innate variabilities among different enterocyte lines which manifested especially in monolayer development were also observed.

Air-Liquid Interface (ALI) culturing condition had proven to have positive effects on monolayer development in vitro. The integrity of the monolayer was able to be maintained up to 14 days due to decreased cellular apoptosis. The enterocytes under ALI condition exhibited increased polarity and adopted columnar morphology which closely resemble the human enterocytes in vivo. The permeability of the ALI cultured monolayer was more physiologically relevant. The ALI condition further demonstrated an effect in enhancing the existing differentiation signal resulted in increased cellular differentiation.

Through co-culture epithelial monolayer with intestinal subepithelial myofibroblast, the cellular apoptosis was further decreased, yielded a well-maintained epithelial monolayer. The additional differentiation signal for goblet cell differentiation was also provided by myofibroblast and was able to be enhanced through ALI culturing condition. It is also confirmed that myofibroblast exerts its effect on intestinal monolayer development via Bone morphogenetic protein (BMP) signaling pathway.

The overall goal of this research was to develop a more physiologically relevant human intestinal epithelium model in vitro. Through combination of ALI culturing condition and co-culture with intestinal subepithelial myofibroblast, a monolayer consisted of polarized, columnar enterocytes with appropriate cellular differentiation was developed.

The dissertation of Po-Yu Lin is approved.

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2019

DEDICATION

This dissertation is dedicated to my family, especially my parents, who have unconditionally supported me along the way.

I would also like to express my gratitude to Anne Lin, who has never given up on me in this journey, helped me through ups and downs with her endless encouragement.

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LIST OF ACRONYMS

ISC	intestinal stem cell
TA cells	transit amplifying cells
MTX	methotrexate
DNA	deoxyribonucleic acid
ISEMF	intestinal subepithelial myofibroblast
DMEM	Dulbecco's Modified Eagle's Media
FBS	fetal bovine serum
PSQ	Penicillin-Streptomycin-Glutamine
EDTA	ethylenediaminetetraacetic acid
HEPES	4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid
EGF	epidermal growth factor
DTT	dithiothreitol
PBS	phosphate-buffered saline
ABAM	antibiotic-antimycotic
TEER	transepithelial electrical resistance
Muc2	mucin 2
DAPI	4',6-diamidino-2-phenylindole
TUNEL	in situ BrdU-Red DNA fragmentation

RNA	ribonucleic acid
Lgr5	leucine rich repeat containing G protein-coupled receptor 5
GAPDH	glyceraldehyde 3-phosphate dehydrogenase
PCR	polymerase chain reaction
H&E	hematoxylin and eosin
PM	proliferation media
DM	differentiation media
mRNA	messenger ribonucleic acid
qRT-PCR	quantitative real time polymerase chain reaction
ECM	extra cellular matrix
Zo1	zonula occludens-1
ALI	air-liquid interface
ATP	adenosine triphosphate
Lyz	lysozyme
CHGA	chromogranin a
Vil1	villin
Slc11a2	solute carrier family 11 member 2
FITC	fluorescein isothiocyanate
MAPK	mitogen-activated protein kinase

BMP4   bone morphogenetic protein 4

CDKN1a   cyclin dependent kinase inhibitor 1a

SMA   smooth muscle actin

CM   conditioned media

BMP   bone morphogenetic protein

PGE2   prostaglandin 2

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CHAPTER ONE

INTRODUCTION

1.1 Background

1.1.1 Physiology of the gastrointestinal track

The intestinal tract is comprised of two segments, the small intestine and the colon. Both segments are comprised of four concentric layers, namely, mucosa, submucosa, muscular layer and serosa Each layer has its own distinctive function. The mucosa is the inner most layer which involves both absorptive and secretory functions. Additionally, in the small intestine, to maximize the surface area for absorption, there are numerous finger-like protrusions known as villi. The villi lies in proximity with invaginations called crypts of Lieberkuhn where the Lgr5+ intestinal stem cells (ISCs), transit amplifying (TA) cells, Paneth cells situate. [1]

One other important role of the intestinal epithelium is its role as an efficient barrier between the environment and our body. Firstly, the single layer of columnar epithelial cells tightly packed together acts as a physical barrier. Secondly, the epithelium is covered with a layer of mucus secreted by Goblet cells which is mostly composed of mucins that both trap antimicrobial peptides and neutralizing secretory IgA antibodies. Furthermore, the mucus layer also prevent the direct contact between the microbiome and the epithelium. [2]

Since the intestine has both secretive and absorptive functions, intestinal epithelium is comprised of several different cell lineages. Most of the cells of the epithelium are enterocytes with absorptive function. The second most cell types would be mucus secreting goblet cells. Intestinal hormones secreting enteroendocrine cells sparsely situated across the villi. Paneth cells not only have antibacterial substances secreting function but are also part of the stem cell niche where they situated inside the crypts, next to ISCs.[3]   The fully differentiated epithelial cells on the villi

are renewed roughly every 5 days. Such high turnover rate is contributed by the stem cells reside at the crypt base. These ISCs give rise to transit amplifying (TA) cells through asymmetric cellular division, which proliferate rapidly as they migrate along the crypt-villus axis. TA cells spend approximately two days in the crypt following progressive differentiation 4 ~ 5 times and emerge onto the villus as mature, functional epithelial cells. [4].

The matured, differentiated epithelial cells that reach the tip of the villus would undergo spontaneous apoptosis and sloughed off into the lumen in approximated 4~5 days. On the other hand, Paneth cells experienced a different fate where they moved toward opposite direction along the crypt-villus axis, toward the crypts. Paneth cells will stay functional a lot longer than other differentiated epithelial cells, around 6 ~ 8 weeks [3], [5]–[8].

1.1.2 Animal model

The main goal of most of the research is to first, understand the mechanism of the disease, either in the molecular level or in the mechanism of the infection, then develop a potential therapy based on the understanding of the disease to mitigate the disease. Human trial would always be the last stage of the therapy development. Animal model would be the first to be used to examine the efficacy and safety of the potential therapy. Although many of these animal models showed similar phenotypes to the ones observed in humans, recent studies in sepsis, for example, show that the molecular mechanisms can be quite different than the mechanisms in humans. This inability of animal model to accurately reflect diseases in human has increasing became a significant hurdle in the path of therapy development. Furthermore, it is likely also the reason to many therapies fail to demonstrate efficacy and safety in human clinical studies while it showed otherwise in animal model. Therefore, there is a great need to develop testing platform which containing primary human cells that could recapitulate specific tissue function in vitro. Researchers have placed a significant amount of focus on developing such platform which could

reflect intestinal functions. One of the reasons to this is that most of the drugs were taken and absorbed via GI track, therefore the intestinal absorption, cell transport, toxicology. studies of probiotics and intestinal interactions between hosts and pathogens has also taken its stride. [9], [10].

1.1.3 Human epithelial colorectal adenocarcinoma cells (Caco-2)

Although intestinal epithelial cells can be isolated, their survival time in vitro is very limited with extensive cell death occurs in a short period of time [11], [12]. Therefore, during the last four decades the transformed, immortalized epithelial cell lines like Caco-2, heterogeneous human epithelial colorectal adenocarcinoma cells, and HT29 have been widely used as intestinal epithelium testing platform. When grown on filter supports, Caco-2 cells will demonstrate a polarized, differentiated monolayer which resemble the intestinal epithelium. However, one major drawback of Caco-2 culture system is that it does not produce mucins. Researchers compensated this issue with co-culturing with HT29 cell line where when HT29 treated with the antimetabolite methotrexate (MTX), they differentiated into mature goblet cells. [2].

Although Caco-2 platform exhibit features that are similar to normal small intestinal epithelial cells, several concerns still exist. Firstly, the research carried out on these cell lines cannot be directly transferred to humans [10] since it is not a normal human cell lines. Secondly, there are many limitations including it required a long time in culture to mature (~ 20 days in culture) and the presence of numerous undefined DNA mutations [12]. Lastly, the absorption rates for are sometimes underestimated in Caco-2 cell culture model when compare to in vivo data [13, p. 29].

1.1.4 Intestinal stem cells

Ever since the confirmation that the different intestinal epithelium cell types are differentiated from a single multipotent stem cell via genetic lineage tracing in late 1980s, researchers have been trying to characterize the stem cell niche that support those multipotent stem cells. Today, the

stem cell niches are well identified. There involved 4 main pathways in maintain the stem cell homeostasis.   Canonical Wnt signaling pathway is a pathway to maintain the stemness and induce the proliferation of stem- and TA cells, it also drives the differentiation of Paneth cells. Notch signaling pathway is another pathway to maintain the stemness of the ISCs. Epidermal growth factor (EGF) signals also plays a role in ISC and TA development. Lastly, bone morphogenetic protein (BMP) signaling pathway is another important factors in affecting intestinal stem cell fate. [1], [3], [5]–[7], [14]–[17].

1.1.5 3D enteroid/organoid culture system

With this advancement in ISCs, researchers start to utilize these stem cells create a more physiologically relevant study platform. Researchers have been successfully regenerating "mini-gut" (organoids) from intestinal stem cell in vitro under the 3D culturing condition while supplementing with various small molecules, growth factors in the culture media to engage in the carious signaling pathways essential to ISC growth. The organoids exhibit proper crypt-villus structure with apical side facing inward along with differentiated enterocytes and Paneth cells [3], [8], [15], [16], [18]–[21]. Sato et al. further examine the potential of using organoids for intestinal tissue repair through transplantation [22].

3D culture models have been proven useful for studying the molecular basis of tissue function and capture signaling pathways and drug responsiveness in some diseases compare to Caco-2 epithelium model. However, there are also come limitations. For example, it is difficult to study single organoids for an extended period of times as they are highly variable in size and and would not maintain in consistent positions in these structures for extended analysis. Another drawback of 3D model rises form its inverted polarity with apical sides facing inwards and difficult to access. With lumen closed off, functional analysis of the intestinal epithelial cells, for example, to quantify transcellular transport, absorption or secretion is technically challenging [23], [24].

1.1.6 2D intestinal monolayer culture system

Upon realizing the inherent limitation of the 3D enteroid culture system, the possibility of deriving a 2D gastrointestinal epithelial monolayer consisted of primary human epithelial cells from ISC rich enteroids started to be explored. Andrew Scott et. al. [25] have demonstrated the feasibility of culturing human intestinal epithelial stem cells as monolayer on Type I collagen and laminin coated tissue culture plate. The stem cells continued to proliferate as a monolayer for 5 days and can be passaged and re-embedded into Matrigel to form enteroids. Other than generating monolayers from 3D cultured enteroids on plastic tissue culture plate, the potential of growing enteroids derived monolayers on a Transwell insert are also being investigated [12], [24], [26]–[28]. The advantage of utilizing a Transwell insert is that it provides access to both apical and basal side of the epithelial monolayer where several studies could be performed. Gaelle Noel et. al. [28] utilizing this system to study the interaction of gastric mucosal surface and host-pathogen interaction via co-culturing gastric monolayer and macrophage at both side of the Transwell membrane. Studies on the infection of Escherichia coli and H. pylori in intestinal mucosal layer were also being investigated [26], [27]. Other than the area of intestinal infection, the immune function of the monolayer such as immunoglobulin A (IgA) transcytosis was also studied utilizing such monolayer culture system [12].

Of all the different studies conducted with Transwell insert culture system, exist a common practice. Commonly, the monolayer would be derived from plating disassociated enteroids onto the Transwell membrane. The cells would then be cultured with media which supplemented with Wnt3a, R-spondin, Noggin to ensure the proliferation of the epithelial cells. Once the full confluency was reached, the Wnt3a, R-spondin would then be removed from the culturing media to induce the differentiation via transforming proliferative cell types to secretive cell types such as goblet cells. This method ensured the monolayer consisting different cells types which would be present in a fully differentiated epithelium before the experiment being conducted. However, after

the monolayer is fully differentiated via this method, the experiments were typically being conducted for up to 72 hours due to the lack of proliferative cell types [12], [24], [26]–[28]. Furthermore, although the polarity of the monolayer was captured via this methodology, the morphology of the monolayer did not exhibit columnar shape [12].

The short experimental window due to the lack of proliferative cell types after differentiation induction and without physiological relevant cellular morphology hinders some studies should they required long term observation such as the interaction of intestinal epithelial and its stromal cells.

With the advantages and shortcomings of the Transwell monolayer culture system, in order to investigate the interaction of intestinal epithelial monolayers and intestinal subepithelial myofibroblast (ISEMF), it would be an important step to first understand the monolayer development and optimize such systems.

1.2 Research objectives

a) To understand the monolayer development in Transwell culture system

b) To investigate new means to further support the maintenance and integrity of the monolayer

c) To study the interaction of the intestinal epithelial monolayer and intestinal subepithelial myofibroblast (ISEMF)

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CHAPTER TWO

THE INVESTIGATION OF DEVELOPMENT OF THE INTESTINAL EPITHELIAL MONOLAYER IN VITRO

2.1 Introduction

Although intestinal epithelial cells can be isolated, their survival time in vitro is very limited with extensive cell death occurs in a short period of time [1], [2]. Therefore, during the last four decades the transformed, immortalized epithelial cell lines like Caco-2, heterogeneous human epithelial colorectal adenocarcinoma cells, and HT29 have been widely used as intestinal epithelium testing platform. When grown on filter supports, Caco-2 cells will demonstrate a polarized, differentiated monolayer which resemble the intestinal epithelium. However, one major drawback of Caco-2 culture system is that it does not produce mucins. Researchers compensated this issue with co-culturing with HT29 cell line where when HT29 treated with the antimetabolite methotrexate (MTX), they differentiated into mature goblet cells. [3].

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With the advantages and shortcomings of the Transwell monolayer culture system, in order to investigate the interaction of intestinal epithelial monolayers and intestinal subepithelial myofibroblast (ISEMF), it would be an important step to first understand the monolayer development and optimize such systems.

2.2 Materials and Methods

Generation of L-WRN condition media

L-WRN condition media was generated in a large batch fashion. L-WRN cells were seeded into 15 cm tissue culture dishes (Corning) with culture media comprise of Dulbecco's Modified Eagle's Media (DMEM, Life Technologies), 10% Fetal Bovine Serum (FBS, Atlanta biologicals), 1X Penicillin-Streptomycin-Glutamine (PSQ, Thermo Fisher) until confluent. Once confluent, G418 (500 µg/mL, Millipore Sigma) and Hygromycin (500 µg/mL, Millipore Sigma) were added into the culture media for cell selection. After 24 hours of selection, the media was switch back to culture media for the surviving cells to again reach confluency. Once the cells were confluent, the cells were disassociated via incubation with 2mL of 0.25% Trypsin-EDTA (Fisher) at 37 °C for 5 minutes and quenched with 5 mL of cell culture media. The cell suspended solution was then collected and centrifuged at 1000 rpm for 5 minutes. The pallets were then expanded into 4 500 cm^2 Triple flasks (Fisher) and cultured with cell culture media until confluent. Once the confluency was reached, the cells were cultured with collection media comprised of Advance-DMEM/F12 (Fisher) and 10% FBS and 1X PSQ. The next day, the supernatant was collected via EMD Millipore Stericup (Fisher) and denoted as Batch 1. The cells were recovered with fresh collection media. The collection process was repeated four times for the subsequent four days. The whole process would result in four batched of the L-WRN conditioned media (L-WRN CM).

Proliferation media

The proliferation media was consisted of 50/50 mix of Advanced DMEM/F12 (Invitrogen) and L-WRN conditioned media (L-WRN CM). The mixture is also supplemented with 1mM N-Acetylcysteine (Sigma), 2mM GlutaMax-1 (Invitrogen), 1mM HEPES buffer (Invitrogen), 10mM Nicotinamide (Sigma), 1X B-27 supplement (Thermofisher), 0.5 µM A83-01 (Sigma), 10 nM Gastrin (Sigma), 50 ng/mL recombinant human EGF (Peprotech), 1X PSQ (Thermofisher) and 10 µM SB-202190 (Sigma).

Isolation of human intestinal crypts

Small intestinal samples were obtained fresh from the Surgical Pathology Department. Tissue was removed from PBS solution and washed multiple times with ice-cold PBS until the solution remained clear. The specimen is placed in a Petri dish containing PBS on ice with the mucosal surface facing upward. Mucosectomy is then performed with surgical scissors and forceps. The mucosa is divided into approximately 1 x 1 cm pieces and washed with cold PBS via vortexing for 30 seconds with three 10-second pulses until the supernatant is clear. These pieces were then incubated in 8 mM EDTA and 1mM DTT solution in PBS for 30 minutes with gentle shaking at 4 ˚C. After the incubation period, the fragments were allowed to settle and the supernatant was discarded. 30 ml of cold PBS is added to the sample, and subsequently vortex for 30 seconds with 3-second pulses. 15 ml of the supernatant was removed and save on ice. Again 15 ml of PBS were added and the process was repeated six times. Six fractions were spun down at 100 g for 2 minutes. The supernatant was discarded. The contents of the pellets were examined under light microscopy. Typically, all fractions were pooled together to increase yield of epithelial crypts. The pooled fractions were then purified using a 100 μm and 70 μm pore filters. The pooled fraction after filtration would be crypts-rich suspended solution

Maintenance of human intestinal stem cells

Every 250 crypts are suspended in 25 μL Matrigel as described in Sato's 3-D Matrigel culture system developed for murine intestines [7] The 25 μL of crypt cell/Matrigel suspension is placed in 48-well plate. Matrigel (Fisher) was allowed to polymerize in the incubator for 15 minutes. 250 μL/well of proliferation medium will then be added to the wells. 10 μM Y-27632 dihydrochloride (Fisher) and 2.5 μM CHIR-99021 (Fisher) were added into the proliferation media for the first two days after passage. The enteroids were maintained in 37°C humidified incubator with 5% carbon dioxide until ideal number of enteroids was reached. The media was changed every two days. At the time of passaged, the enteroids containing Matrigel buttons were dislodged with P1000 pipettes and along with media, collected into 1.5 mL Eppendorf tubes. The pellets were acquired

via microcentrifuge with three 3-second pulses. The Matrigel containing pellets were then digested with 500 μL of TrypLE Select disassociation reagent (Thermo Fisher) in 37°C water bath for 5 minutes. The disassociation reagent was then quenched with DMEM with 10% FBS and 1X AMAB (Life Technologies). After quenching, the enteroids were further broken down mechanically via pipetting the content with P1000 pipettes repeatedly. The pellets were then acquired via microcentrifuge with three 3-second pulses. The pellets were then resuspended with desirable amount of Matrigel and re-seeded into a new tissue plate.

Generation of intestinal monolayer

A.   *Collagen IV preparation and coating*

Human collagen IV (Sigma) was reconstitute in 100 mM acetic acid solution into 1mg/mL concentration stock solution. The working collagen IV solution (30 μg/mL) was prepared by diluting the stock solution with sterile deionized water. The inserts would then be covered with collagen IV working solution and incubated in 37 ˚C incubator for at least 2 hours ensuring successful coating.

B.   *Generation of intestinal monolayer*

Transwell culturing method was adopted from Julie In et al [19] The 24-well Transwell inserts (Corning 3414) with polycarbonate membrean were coated with collagen IV (30 μg/mL) prior cell seeding.

The enteroids-containing Matrigel buttons were scraped off from the tissue culture plate with P1000 pipettes and washed with 0.5 mM EDTA/PBS (Sigma). After pelleting the content via centrifuging at 1000 rpm for 5 minutes, the Matrigel/enteroids were then disassociated chemically with 0.25%/0.5mM Trypsin/EDTA in 37 ˚C water bath for 5 minutes. After quenching with DMEM/10% FBS/1X ABAM, P1000 and P200 pipettes were used to break apart the

15

enteroids mechanically, ensuring the enteroids were disassociated into single cells. Cell containing solution were then pass through 40μm cell strainer before centrifuging at 1000 rpm for 5 minutes.

cell pellets were resuspended with proliferation media supplemented with Y-27632 dihydrochloride and CHIR-99021. The cells were seeded into the the collagen IV coated Transwell inserts at a seeding density of 750k/cm^2.

The monolayers would be cultured in proliferation media with 100 μL in the insert and 600 μLin the well with media change every 2 days.

TEER measurement

TEER measurement was done with Epithelial Volt/Ohm meter (World Precision Instrument). Prior the measurement, 250 μL of fresh media was added into the insert and 700 μL of fresh media was added into the well. STX2 electrode (World Precision Instrument) was sterilized with 70% ethanol followed by washing with the PBS. When measuring, hold STX2 electrode straight up with short end in the insert and long end in the well. The reading on the meter would be in the unit of ohm (Ω). To convert, subtracted the reading with the reading of a blank insert, then multiply by the area of the insert to acquire the unit of Ωcm^2.

Immunohistochemistry

A.   *Sample preparation for histology*

The monolayers were fixed by adding 200 μL of 10% buffered formalin (Fisher) into the inserts for 30 minutes in room temperature. The monolayers were washed with PBS twice after the fixation. The samples needed to be embedded within Histogel (Fisher) in an upright fashion. Liquified the Histogel by warming it up to 65°C and was kept warm for it to remain at liquid state. The polycarbonate membrane was carefully cut off with scalpels. The membrane was them cut into

four strips with razor blades and stacked one on top of each other. Place a lid of the 10 cm diameter petridish on an ice block and, with transfer pipette, drops couple droplets of liquified Histogel onto the lid for form a button. Once the Histogel button was solidified due to the drop of the temperature, cut a slit on the button and place the membrane strip into the slit of the Histogel in an upright position so when sectioning, the cross section of the monolayer could be observed. The Histogel button was then covered with fresh Histogel ensuring the strip was covered with Histogel. The button was then leaved on the ice block for 15 minutes before transferring into -20 °C for 10 minutes. Lastly, the strip containing Histogel button was then transferring to the histology cassette and stored in 70% ethanol before submitting to the Stanford Human Pathology/Histology Service Center for paraffin embedding, sectioning and Hematoxylin and Eosin staining (H&E).

B.   *Immunostaining of differentiation marker*

Unstained slides were sectioned at 4 µm thickness with 2 sections per slide. Slides were washed with xylene for 5 minutes twice to remove the paraffin wax. The sections were then rehydrated with progressive decreasing concentration of ethanol in water from 100% to 70% for 2 minutes and finished with 5 minutes incubation in deionized water. Antigen retrieval was done by incubate the slides in 1X Antigen Retrieval Citra Solution (Fisher) for 15 minutes at 100 °C followed by 20 minutes cooling in cold water bath.   Samples were then permeabilized with 0.5% Triton-X for 5 minutes and washed with PBS-Tween twice afterwards. A hydrophobic barrier was created with PAP pen (Abcam) before covering the sections with blocking solution containing 2% bovine serum albumin and 5% Normal goat serum for 1 hour in room temperature to prevent non-specific staining. Following blocking, primary antibody working solutions with primary antibody diluted in blocking solution at a ration which is antibody specific was added on top of the designated sections. The sections were incubated at 4 °C overnight. Primary antibodies used would be Muc2 (Santa Curz), E-cadherin (Santa Cruz, abcam), ki67 (Santa Cruz), villin (Santa Cruz). Next

day, the slides were washed with PBS-Tween three times to wash away excess primary antibody before adding secondary antibodies which was diluted in PBS-Tween at 1:50 ratio on top of the sections. For the secondary antibodies, Alexafluor 488 goat anti-mouse, Alexafluor 488 goat anti-rabbit, Alexafluor 594 goat anti-mouse or Alexafluor 594 goat anti-rabbit were used depends on the primary antibodies used at 1:200 ratio. Excessive secondary antibodies were washed away with three PBS-Tween wash before adding mounting media with DAPI (Fluoroshield with DAPI, Sigma) was added. Fluorescent images were taken with Olympus microscope with CellSens software (Olymous).

C.   *TUNEL assay*

The In situ BrdU-Red DNA Fragmentation (TUNEL) assay was performed following the protocol provided by the vendor (ab66110, Abcam) with the exception of 0.5% Triton-X instead of protease K treatment. The samples were counterstained with DAPI at the end.

D.   *Quantification of ki67 and TUNEL*

Immunofluorescent pictures of each samples with ki67 staining and TUNEL assay treatment were taken. Three randomly chosen areas of each slides were taken. Positive ki67 cells were only counted when ki67 and DAPI signals were colocalized. TUNEL positive cells were only counted when its signal was colocalized with DAPI. (n=3 for 3 independent experiments)

Analysis of RNA expression

RNA extraction was described in a previous publication [21] Primer and probe combinations were purchased from Applied Biosystem (Taqman Expression Asssay, Hs00969422_m1(LGR5), Hs02758991_g1(GAPDH)). RT-PCR was performed in accordance with the description in a previous publication [21] with ABI 7900HT Fast Real Time PCR system. Cycle numbers of all

samples were normalized to GAPDH with human intestinal (whole bowel (WB) serving as control tissue.

Statistics

Differences between groups were evaluated via Student's t-test. A p-value < 0.05 was considered as statistically significant.

2.3 Result

Comparison of monolayer cultured with proliferation media (PM) and differentiation media (DM)

The picture showed H&E results of epithelial monolayer on top of Transwell insert membrane cultured in proliferation media and differentiation media (Fig. 2-1). The results showed that the monolayer was able to maintain the integrity until day 14, comparing to the monolayer cultured in differentiation media. The cells showed sign of apoptosis and detached on day 14 when cultured in differentiation media.

The reason for this is that although differentiation media can induce cellular differentiation through transforming proliferative cell lineage into differentiated cell lineage, such as goblet cells, the loss of proliferative cells results in the monolayer to lose its integrity through lack of cellular replacement.

The observation of variabilities of the monolayers derived from different enterocyte lines

The phalloidin / DAPI staining of the monolayers generated from two different enterocyte lines showed little difference when examining at top-down fashion (Fig. 2-2) The differences between the two in terms of the integrity of the monolayer, its cellular morphology and polarity was not apparent.
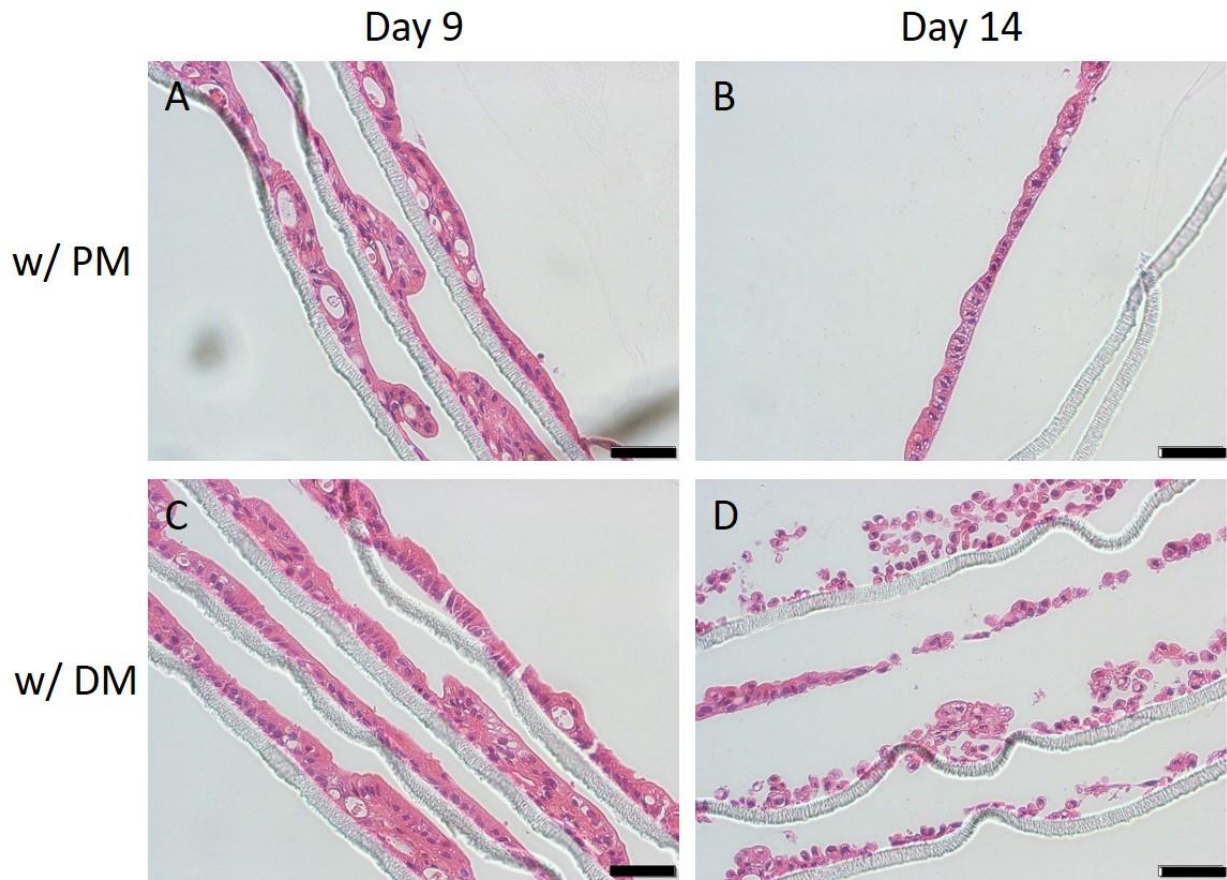
Figure 2-1: Hematoxylin and eosin (H&E) histology of epithelial monolayer on Transwell Inserts.

Comparison of monolayer cultured in Proliferation media (PM) (A)(B) and monolayer cultured in differentiation media (DM) (C)(D).

The lighter color strips beneath the monolayer is the porous membrane of the Transwell inserts.

Scale bar: 50 µm
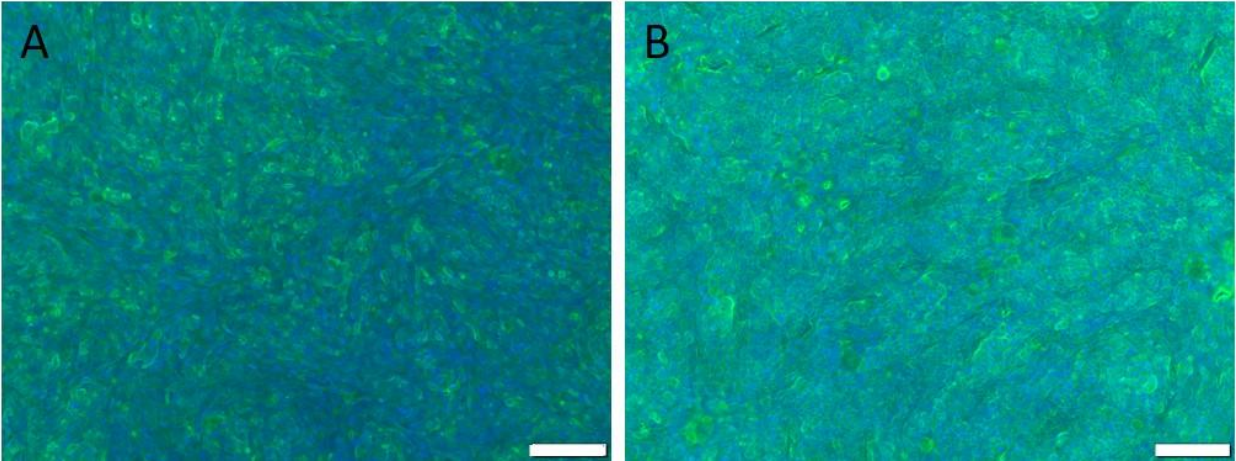
Figure 2-2: Alexa Fluor 488 Phalloidin and DAPI staining on the monolayer derived from two

different enteroid lines

Scale bar: 50 µm

To better establish a monolayer that could maintain the integrity long-term, it was imperative for us to first understand the development of the intestinal epithelial monolayer in proliferation media. We found that there exists an intrinsic variability among different enterocyte lines and this variability could be observed here. The monolayer developed from one enterocyte line demonstrated a homogeneous and well-organized structure up to day 14 (Fig. 2-3). On the other hand, one line developed into a well-organized monolayer at the early time point, but rapidly lose the integrity after day 9. Other two lines further showed this phenomenon in which they lose the integrity at the later time point as well even though they were all cultured under same culturing condition (Fig. 2-4).

Transepithelial electrical resistant (TEER) measurement

The variabilities among different monolayers developed from different enterocyte lines also reflect on the TEER measurement (Fig. 2-5). The variability persisted after the media switch. This poses a potential problem when using TEER as a parameter to examine the effect of a factor on multiple cell lines simultaneously.

*Immunostaining of differentiation markers on the enteroid derived monolayer*

The monolayer developed from this enterocyte line, isolated from human duodenum, would be used as a main study platform since the morphology of the monolayer went from organized to disorganized within reasonable time frame for the study (Fig. 2-6).

To examine the differentiation of the monolayer under proliferation media, we examined a panel of differentiation markers including Muc 2 for Goblet cells, villin for enterocytes as well as the presence of brush border at the apical side of the differentiated enterocytes (Fig. 2-7). From the immunostaining results, it had positive e-cadherin signal, suggests that the monolayer cultured with proliferation media exhibit cell-cell contact of the epithelial cells. However, it showed negative of the Muc2 staining, suggesting the lack of the differentiated goblet cells. Although it showed
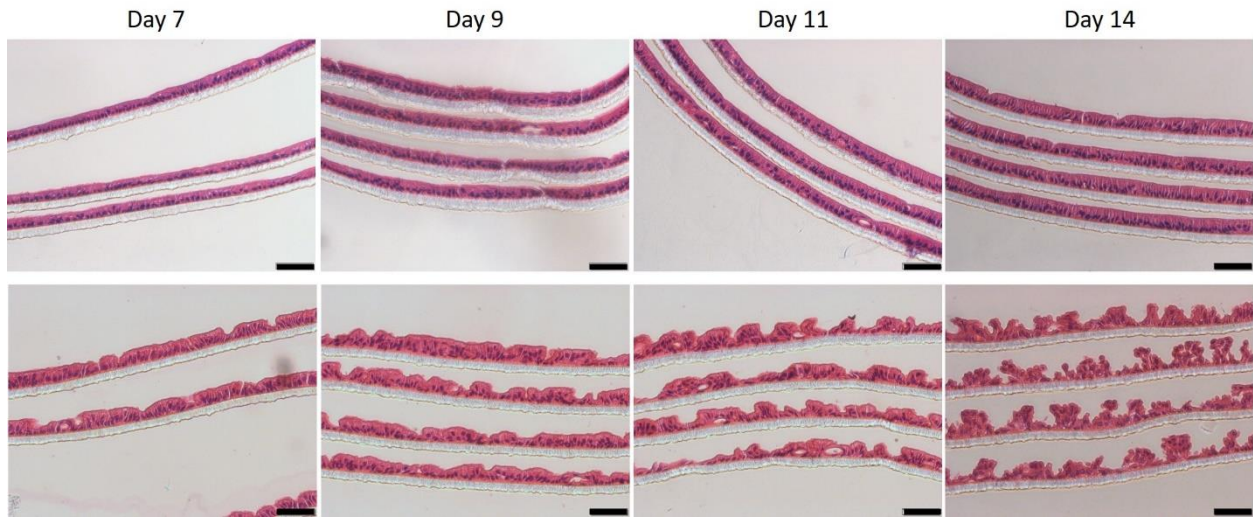
Figure 2-3: H&E histology from day 7 to day 14 of monolayers derived from two different

enterocyte lines (bottom: enterocyte line 1 and top: enterocyte line 2)

Scale bar: 50 μm
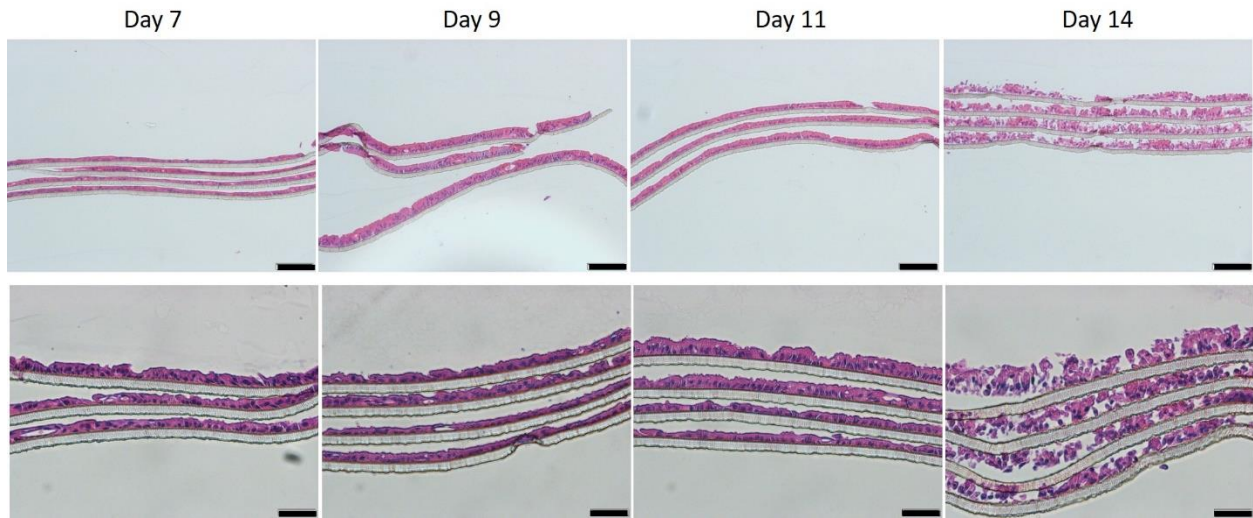
Figure 2-4: H&E histology from day 7 to day 14 of monolayers derived from two different enterocyte lines (enterocyte line 3 and enterocyte line 4)

Scale bar: 50 µm
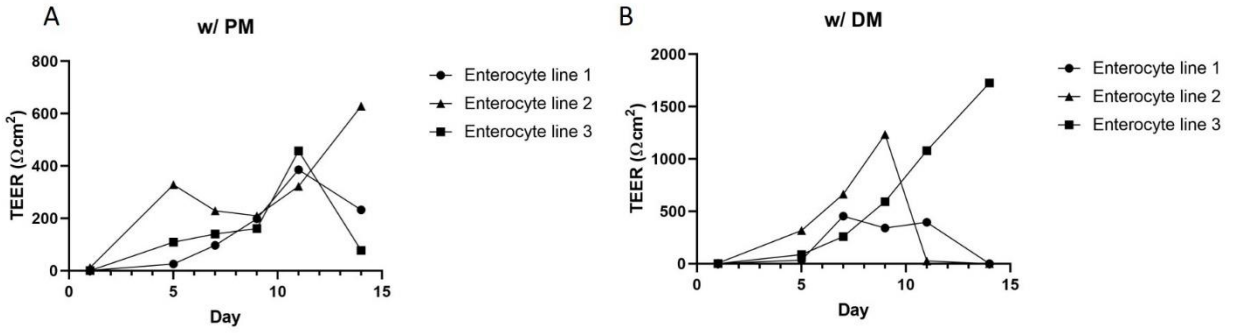
Figure 2-5: TEER analysis on 3 monolayers derived from 3 different enterocyte lines.

Monolayers were cultured with PM (A) and DM (B)
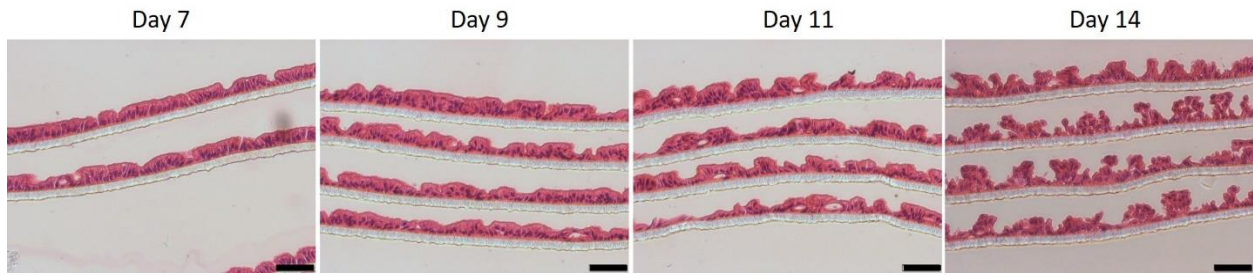
Figure 2-6: H&E histology of the monolayer derived from enterocyte line 1

Figure 2-7: Immunofluorescence of cultured epithelial monolayer from day 7 to day 14. Monolayer was cultured in proliferation media (PM). E-cadherin confirms the epithelial cell lineage. Monolayer lacked Muc2, a marker for goblet cells. No co-localization of Villin at the apical side of the monolayer suggested no formation of brush border.

Scale bar: 50 μm

positive on villin signal, the red signal did not localize at the apical side of the cells, demonstrating the lack formation of brush border at the apical side of the enterocytes. The under-differentiation of the monolayer was expected when culturing with proliferation since multiple active factors in the media were specifically used to promote the stemness and inhibit the differentiation, namely, Wnt3a, r-spondin 2, noggin and other small molecular inhibitors.

Immunostaining of ki67 and TUNEL assay and its quantification

To further study the development of the monolayer, the proliferation and cellular apoptosis of the monolayer was examined (Fig. 2-8). The results showed positive for proliferation marker, ki67, which colocalized with nuclei staining, DAPI, from day 7 to day 11. It suggested that the monolayer had proliferative ability throughout the experiment. However, it also showed positive for cellular apoptosis to great extant throughout the experiment.

From the quantitative analysis of both proliferative cells and apoptotic cells (Fig. 2-9), the averaged proliferative cells remained between 10% and 20% from day 7 to day 14. On the other hand, apoptotic cells remained at over 50% throughout the experiment.

Immunostaining of ki67 and TUNEL assay was performed on the human intestinal histological sections and quantified (Fig. 2-10). Ki67 positive cells co-localized with nucleus were mostly limited in the crypt region where the proliferation take place. On the other hand, apoptotic cells were most prominent at the tips of the villi.

The comparison of ki67 positive cells and apoptotic cells between in vitro cultured monolayers with human intestinal histology sections showed that there were approximated two-fold more of the apoptotic cells than normal human intestine (Fig. 2-11).
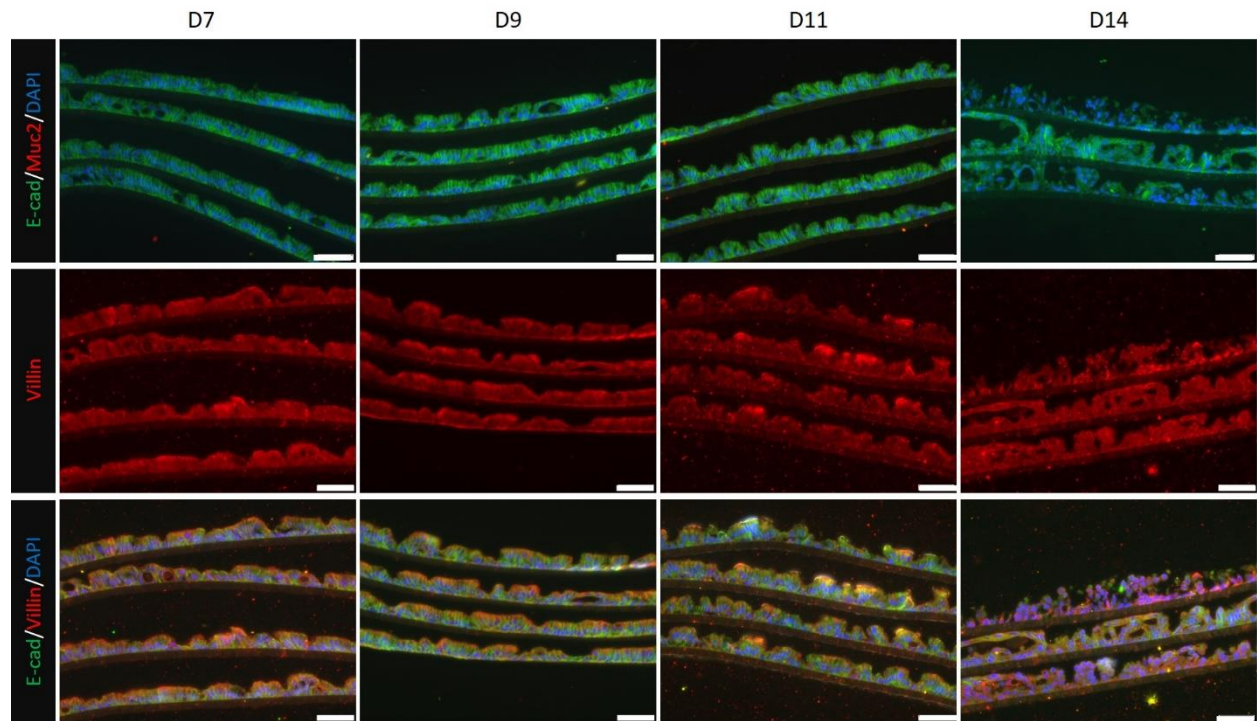
Expression of mRNA in enteroid derived monolayer and enteroids
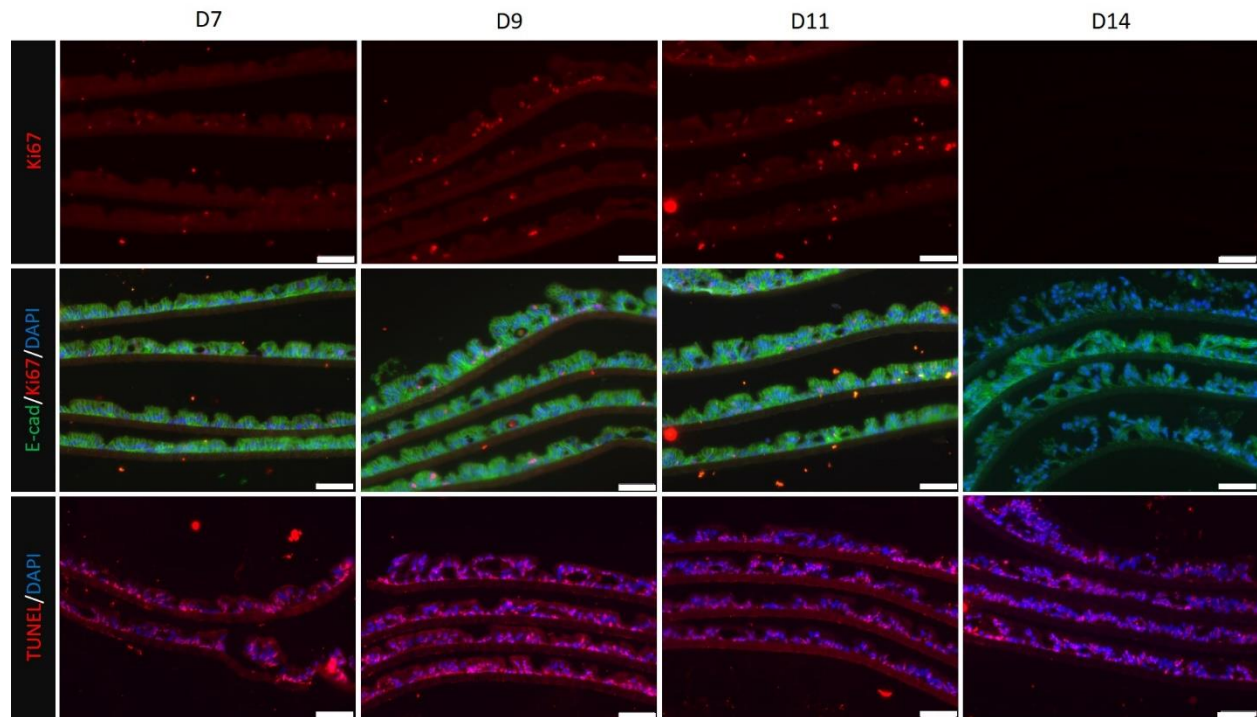
Figure 2-8: Immunofluorescence of cultured epithelial monolayer from day 7 to day 14.

Monolayer was cultured in proliferation media (PM). Positive Ki67 confirms the presence of

proliferation cells. TUNEL positive nuclei confirms the presence of DNA fragment that was

undergoing apoptosis.

Scale bar: 50 μm
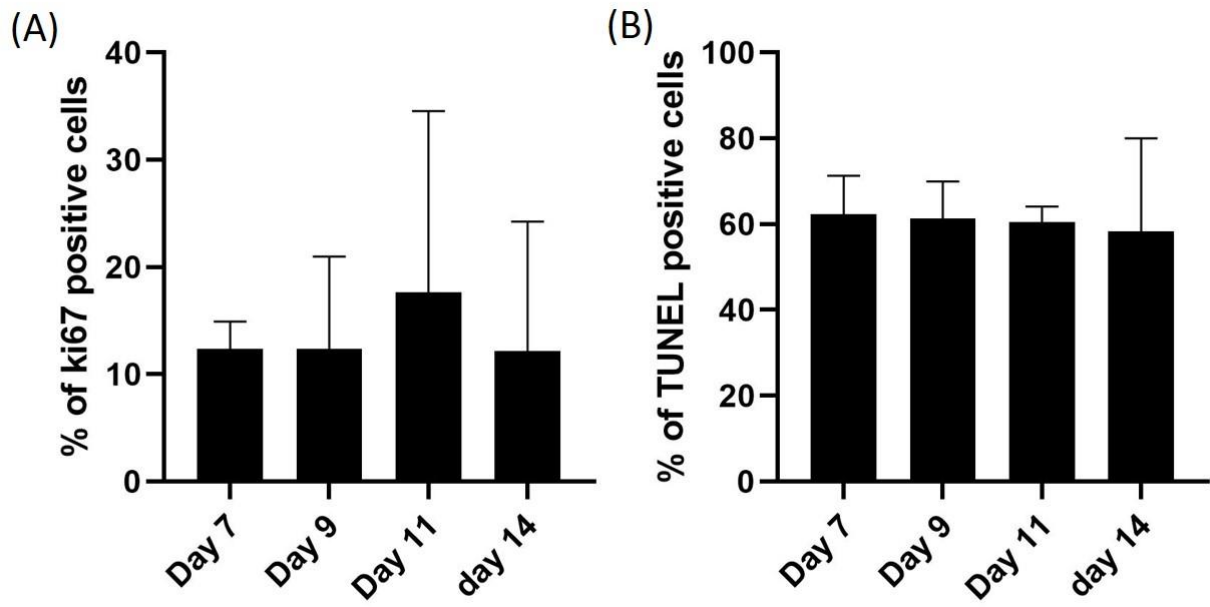
Figure 2-9: Quantification of ki67 positive cells (A) and TUNEL positive cells (B)

There were no statistical differences throughout the experiment. (n=3)
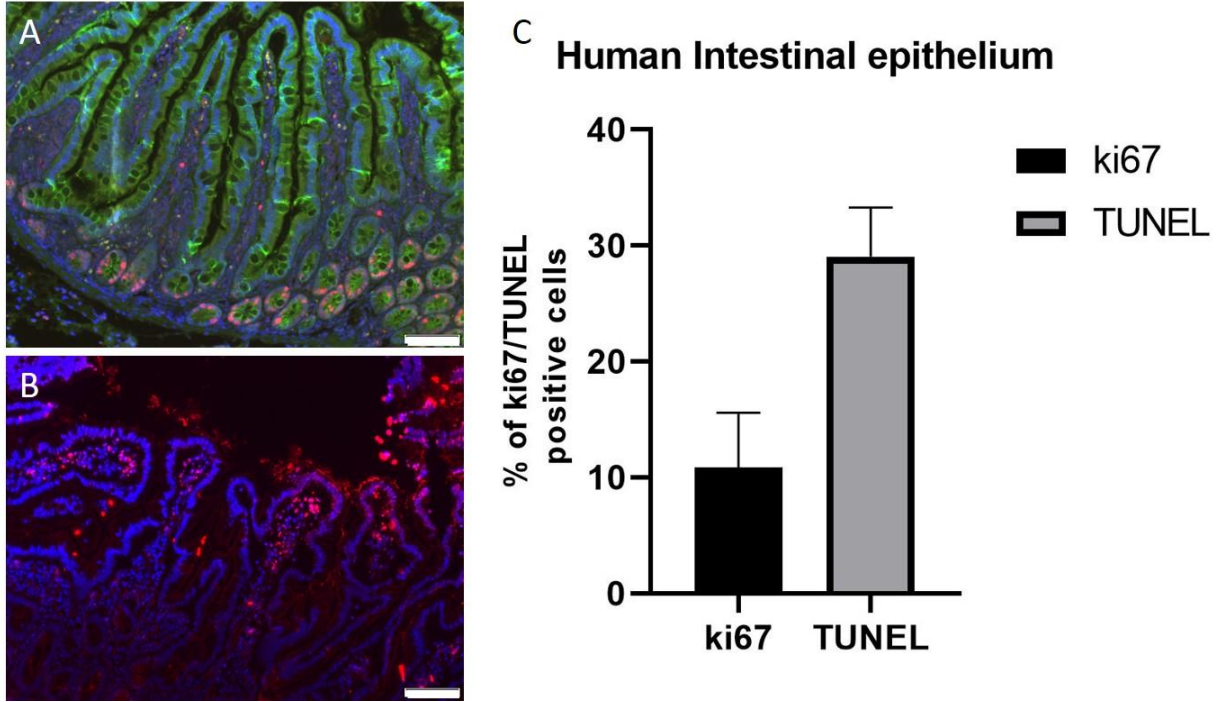
Figure 2-10: (A) Immunofluorescence of ki67 on human intestine section. Ki67 positive (red) cells can be seen limited in the crypts. (B) TUNEL assay on human intestine section. Apoptotic cells (red) largely situated at the tip of the villi. Scale bar: 100 µm

(C) Quantification of ki67 positive and TUNEL positive cells. (n=3)
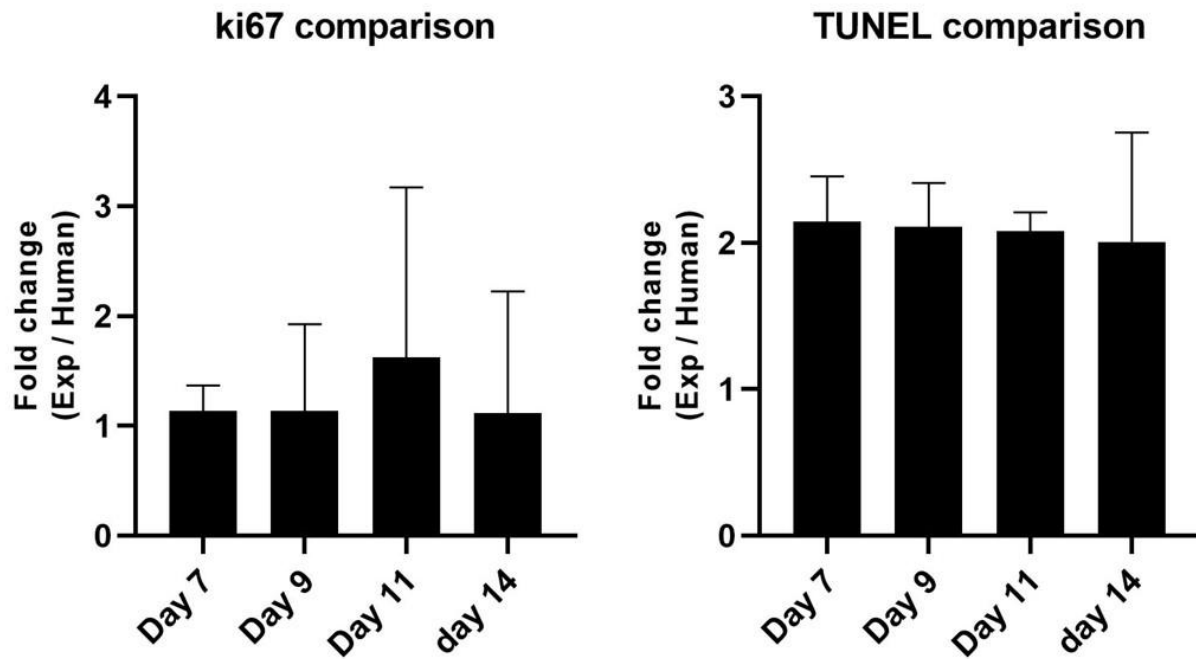
Figure 2-11: Quantitative comparison on ki67/TUNEL between in vitro cultured monolayer and human intestinal epithelium

With positive staining of ki67, one wonders the identity of the ki67+ cells. Lgr5 levels was examined through qRT-PCR (Fig. 2-12). Lgr5 is a stem cell marker of the ISC, therefore, this marker was used intended to differentiate between proliferative ISC and proliferative transit amplifying cells. The results showed an overall low relative expression level of Lgr5, and it has been low at day 0 which denoted the Lgr5 level in enteroids that were used to generate the monolayer for the analysis. The result on day 0 suggested that the enteroids used have already experienced a decrease in Lgr5 level prior being seeding onto the transwell inserts.

With the result from PCR mentions previously, we looked at the Lgr5 level of the enteroids cultured in Matrigel through passages and examine the rate of decrease of Lgr5 in intestinal enteroids in culture (Fig. 2-13). The result showed that the relative expression of Lgr5 consistently decreased from Day 1 through day 5. On day 5, the expression level is significantly low. However, we also observed the "re-set" phenomenon of the Lgr5 expression right after passaging. Through passaging, the Lgr5 expression seems to re-set back to a similar level relative to human intestine.

With the sharp decrease of the Lgr5 expression in culture from day 1 to day 5, we wonder whether this is one of the reasons which contributes to the monolayer to take on a nonhomogeneous, disorganized morphology since, most of the time, the monolayers were generated from enteroids cultured for 5 days in Matrigel. Therefore, the morphology of the monolayer generated from the enteroids cultured in Matrigel for 3 days when there was still certain level of Lgr5 expression was examined (Fig. 2-14). However, the result showed a similar disorganized monolayer morphology as before, suggesting that the low expression level of Lgr5 might not be a factor that results in the loss of integrity of the monolayer at the later time.

Verification of the presence of Lgr5+ cells in the monolayer in vitro

One thing to wonder is that even though the Lgr5 expression was relatively low, the monolayer could still be disassociated and re-seeded back into Matrigel (Fig. 2-15). The disassociated

Lgr5
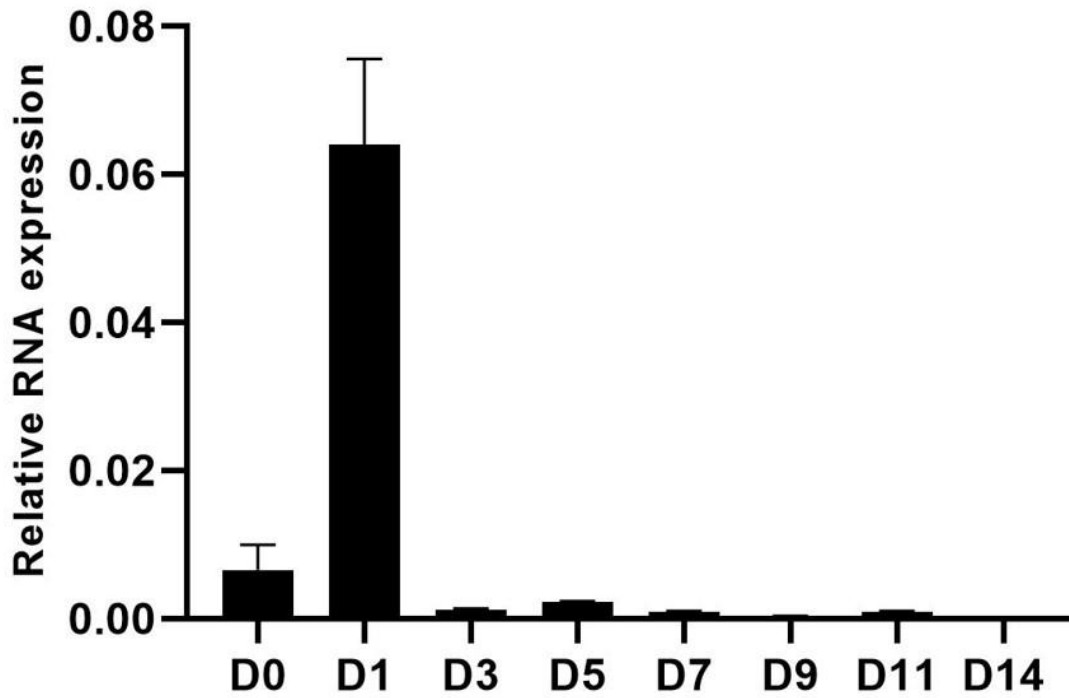
Figure 2-12: Relative RNA expression of Lgr5 of the monolayers via qPCR. The expression is relative to human whole bowel. D0 denoted the RNA collected from the enteroids right before the experiment. (n=3)
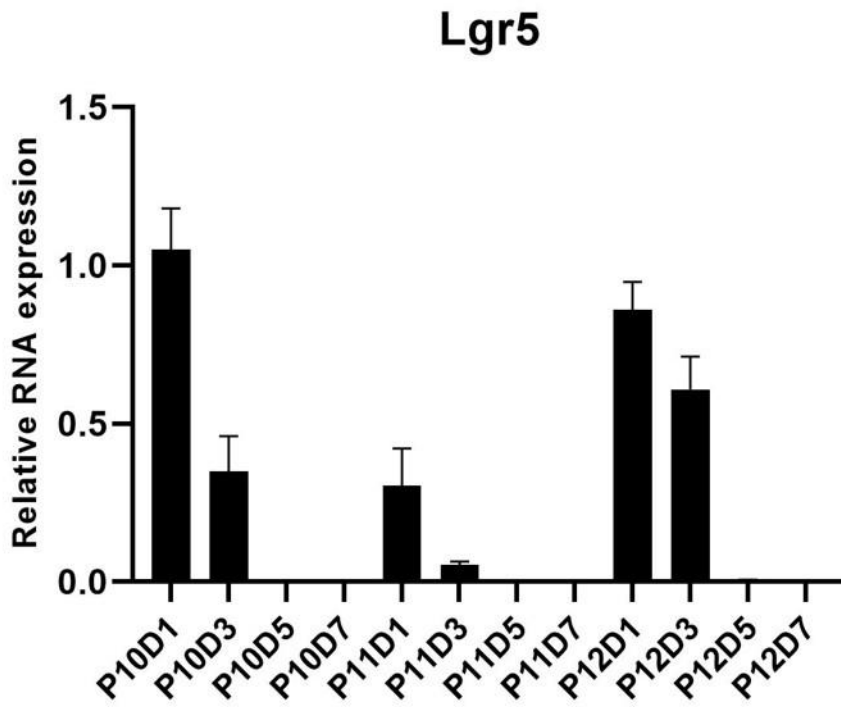
Lgr5

Figure 2-13: Relative RNA expression of Lgr5 of the enteroids via qPCR. The enteroids were cultured from P10 to P12. The expression is relative to human whole bowel. (n=3)
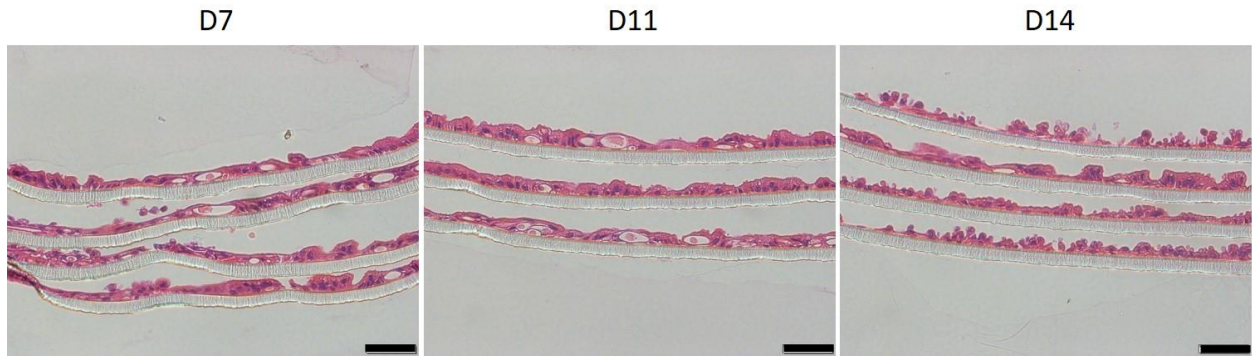
Figure 2-14: H&E histology of the monolayer derived from enterocyte line 1 which had been cultured for 3 days in Matrigel

| D0 | ReD1 | ReD5 | ReD9 |
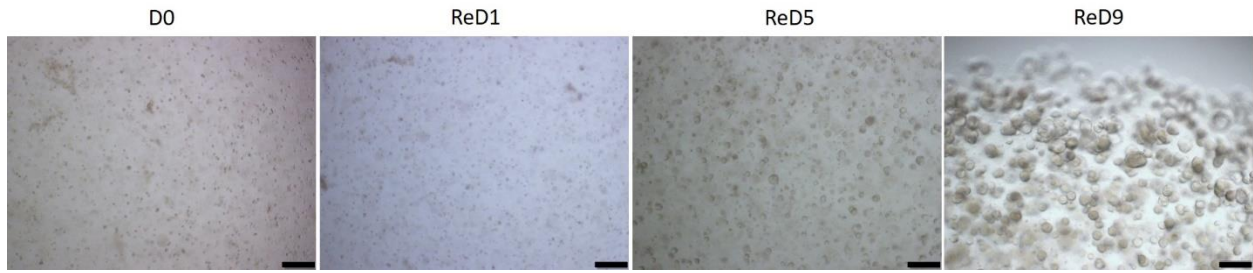
Figure 2-15: After 5 days of cultured on Transwell insert as monolayer, the dissociated monolayer was able to survive and proliferate after reseed back into Matrigel up to 9 days (ReD0 ~ ReD9). Scale bar: 200 μm

epithelial cells were able to survive and proliferate up to 9 days within Matrigel after being cultured on the Transwell inserts as monolayer for 5 days. The picture showed the development of enteroids from the start of the experiment (before being plated into the Transwell insert, D0) to reseeded back into Matrigel for 9 days (ReD1 ~ ReD9). The enteroids increased in numbers and sizes after being reseeded.

Furthermore, the re-seeded enteroids could not only proliferated but also could be passaged, which further confirmed the presence of the Lgr5+ stem cells (Fig. 2-16).

Upon examining the Lgr5 expression of the enteroids from D0 to ReD9 (Fig. 2-17), it showed a similar trend as before. The decrease of Lgr5 expression could be seen after being cultured 5 days as monolayer, then it increased after dissociation and being reseeded back into Matrigel, a process similar to passaging, hence the phenomenon of resetting Lgr5 expression after passaging was captured.

The effect of exogeneous R-spondin 1 on enteroid and monolayer development

The effects of adding additional recombinant R-spondin1 into the enteroid culture and monolayer culture are investigated. R-spondin1 is a well-known agonist of canonical Wnt signaling pathway. Through adding recombinant R-spondin1 into culture, we are interested to see if the Lgr5 expression could be maintained or elevated and whether if it would have positive effect on monolayer development. The results yielded no statistical difference in the Lgr5 expression with or without the addition of recombinant R-spondin1 in enteroid culture (Fig. 2-18).

Upon further examine the difference in monolayer development between with or without addition of recombinant R-spondin 1, no significant difference could be seen after comparing H&E results of two monolayers (Fig. 2-19).

The investigation of the variabilities between two enterocyte lines
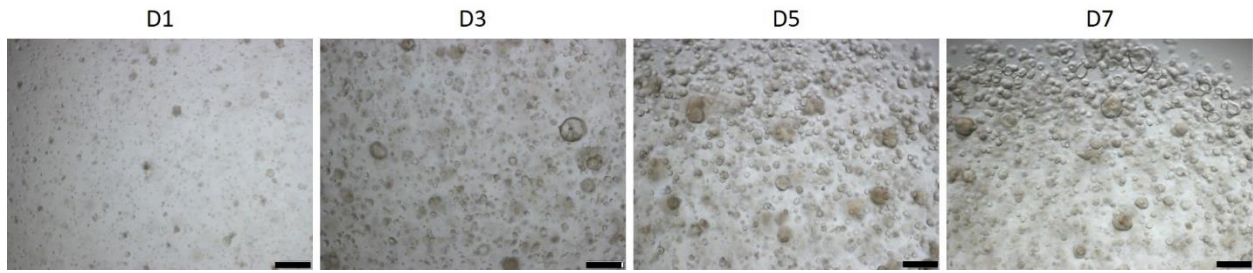
Figure 2-16: After being re-seeded back into Matrigel for nine days, the enteroids could be further passaged. These bright field pictures showed that the enteroids could proliferate after being passaged. This result further confirmed the presence of the Lgr5+ stem cells. Scale bar: 200 µm
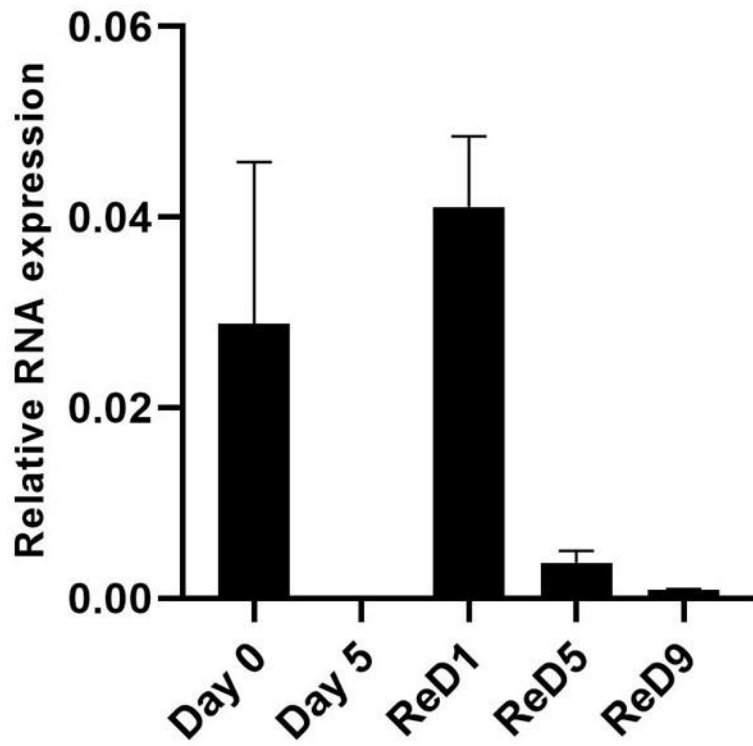
Lgr5

Figure 2-17: Relative RNA expression of Lgr5 throughout the experiment. Lgr5 level of the monolayer decreased significantly after 5 days on the Transwell inserts (Day 5). After the monolayers were dissociated and re-seed back to Matrigel, Lgr5 expression decreased from day 1 to day 9 (ReD1 ~ ReD9). The expression is relative to human whole bowel. (n=3)
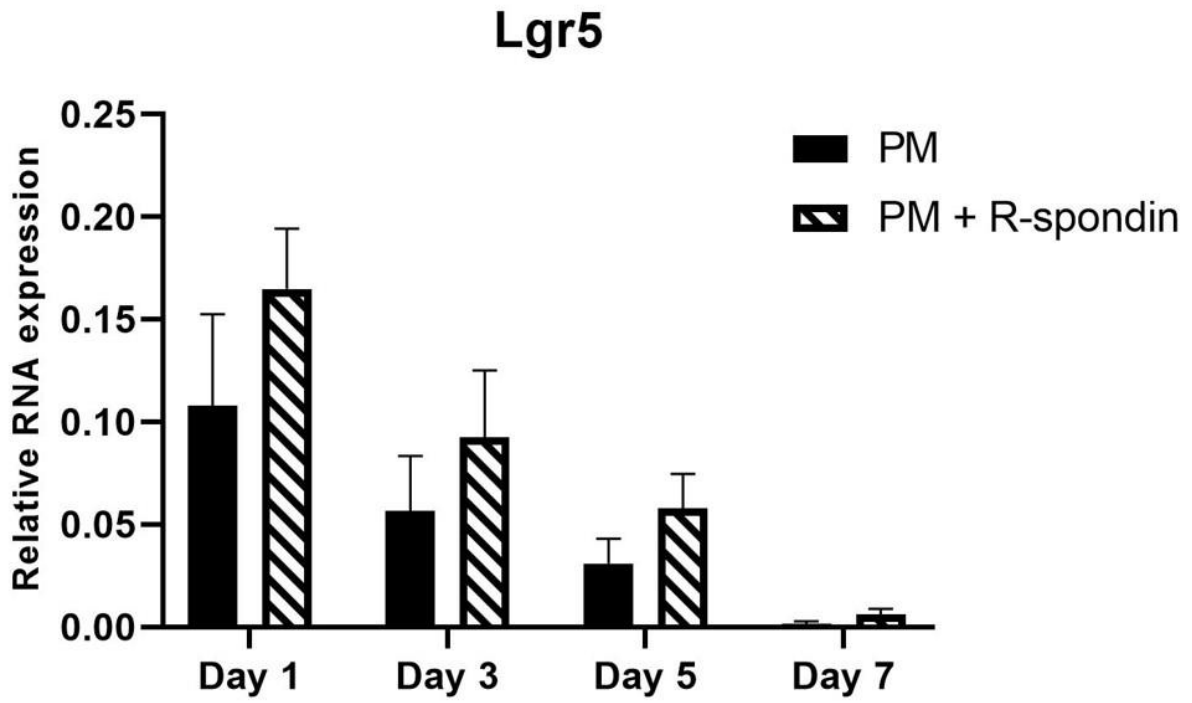
Lgr5

Figure 2-18: With additional recombinant R-spondin1 added into the culture, relative RNA expression of Lgr5 showed not significant difference.
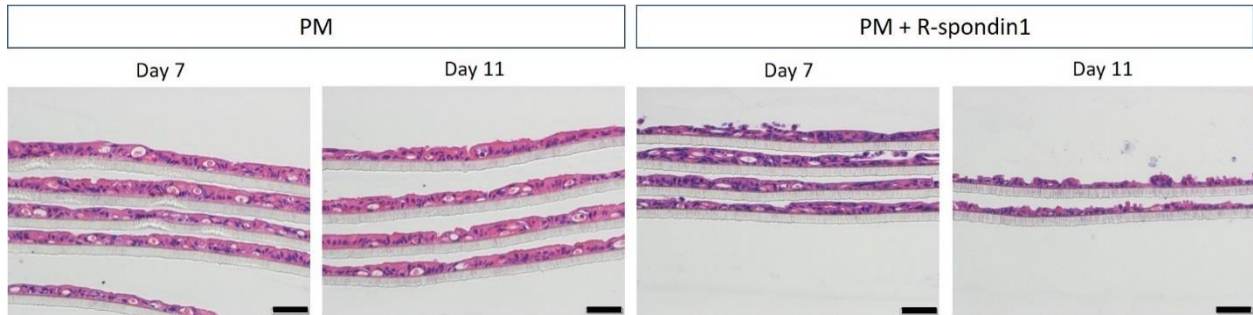
Figure 2-19: H&E histology showed difference in the monolayer morphology between the one with or without recombinant R-spondin1. No significant difference in the morphology between the two monolayers could be observed. Scale bar: 50 µm

The monolayer derived from enterocyte line 2 has consistently demonstrated a much more homogeneous and organized morphology (Fig. 2-20). The monolayer from this enterocyte line was also able to maintain such organized morphology up to day 14. The picture here showed a drastic difference in the cellular morphology of the monolayers from 2 different enterocyte lines cultured under the same condition.

With such a drastic difference in the morphology of the monolayers, the enteroids that had been cultured in Matrigel exhibit no observable difference between the two lines (Fig. 2-21).

Furthermore, despite the Lgr5 expression level of the enterocyte line 2 was significantly higher at the early time point (Fig. 2-22), it also has experienced a similar fashion of steady decrease within the passage with enterocyte line 1. This result suggests that the level of Lgr5 expression did not contribute the variability in the development of the monolayer.

To further explore the difference between the monolayer from these two lines in an effort to determine the key factor that contributes to such a difference, the difference in the cellular apoptosis between the two was examines (Fig. 2-23). Through TUNEL assay, it seems that the monolayer derived from enterocyte line 2 experience less cellular apoptosis than the one from enterocyte line 1.

Through quantitative analysis of ki67 positive cells and apoptotic cells (Fig. 2-24), there are no statistical differences between the two monolayers, they showed similar percentage of the ki67 positive cells as well as the apoptotic cells.

When comparing the quantitative results of ki67 and apoptotic cells from two monolayers with the results from human intestine, no significant difference could be seen (Fig. 2-25).
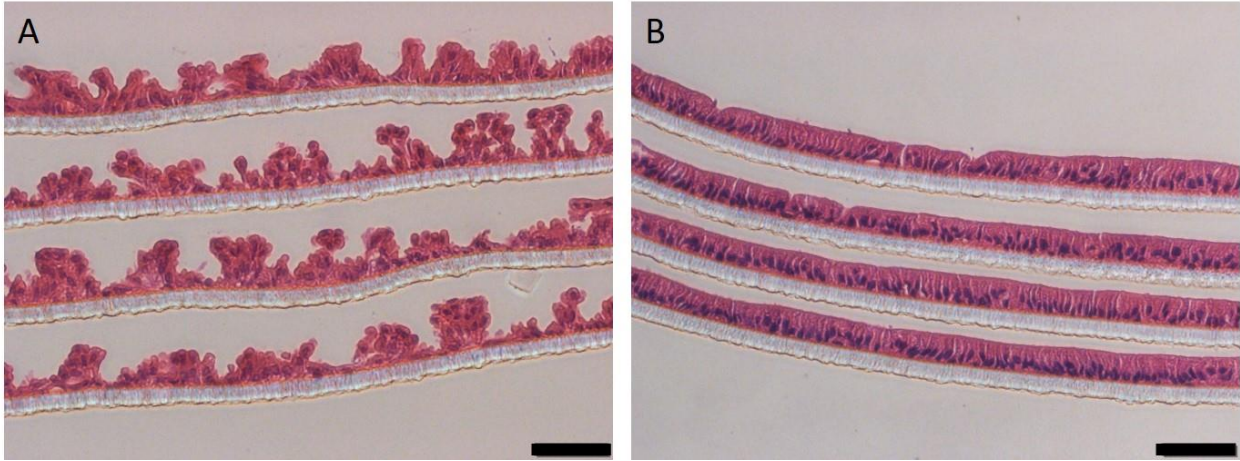
Figure 2-20: H&E histology of the monolayers derived from two different enterocyte lines.

One from enterocyte line 1 (A) and the other form enterocyte line 2 (B). Scale bar: 50 µm
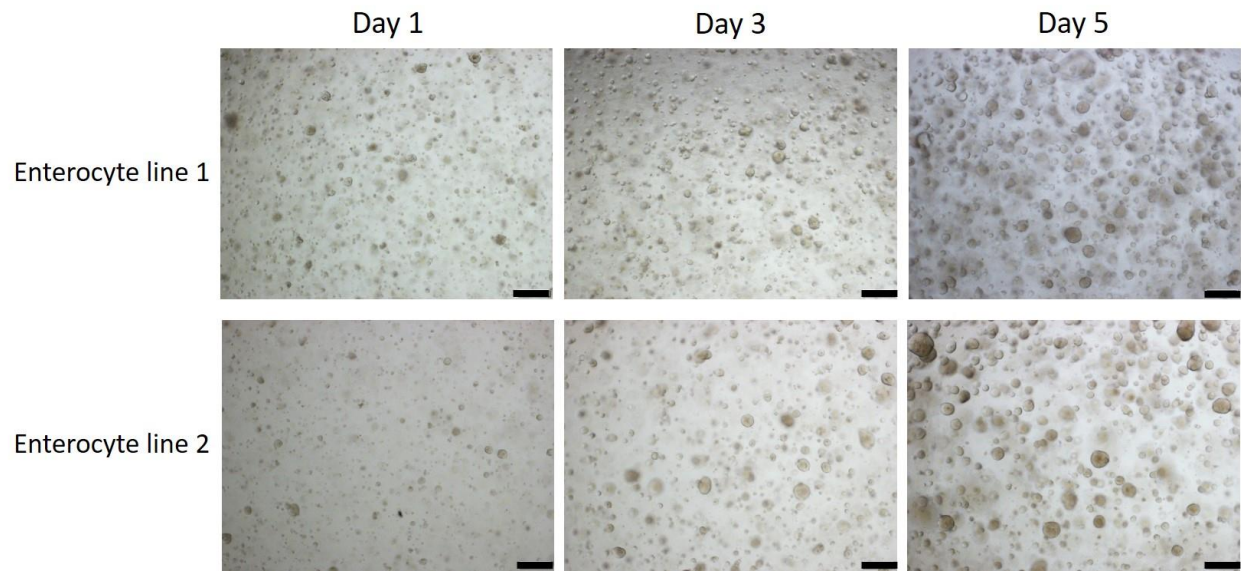
Figure 2-21: Bright field pictures of two enteroids culturing in Matrigel. Scale bar: 200 µm
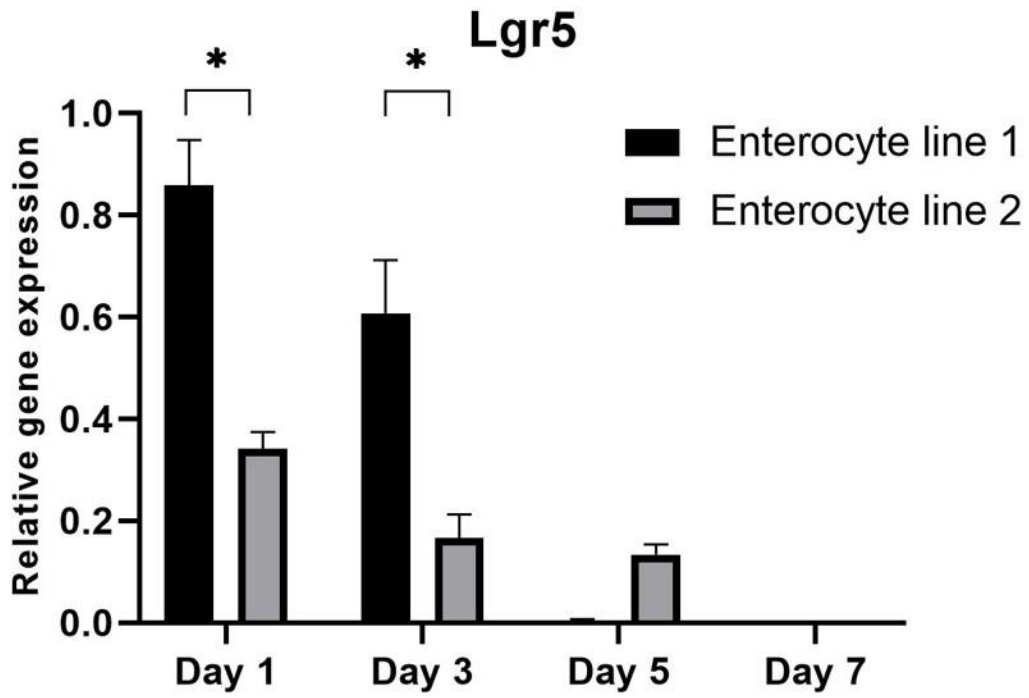
Figure 2-22: Comparison of relative RNA expression of Lgr5 between 2 enterocyte lines. Both lines show similar decreasing trend. The expression is relative to human whole bowel. (n=3) (* p < 0.05)
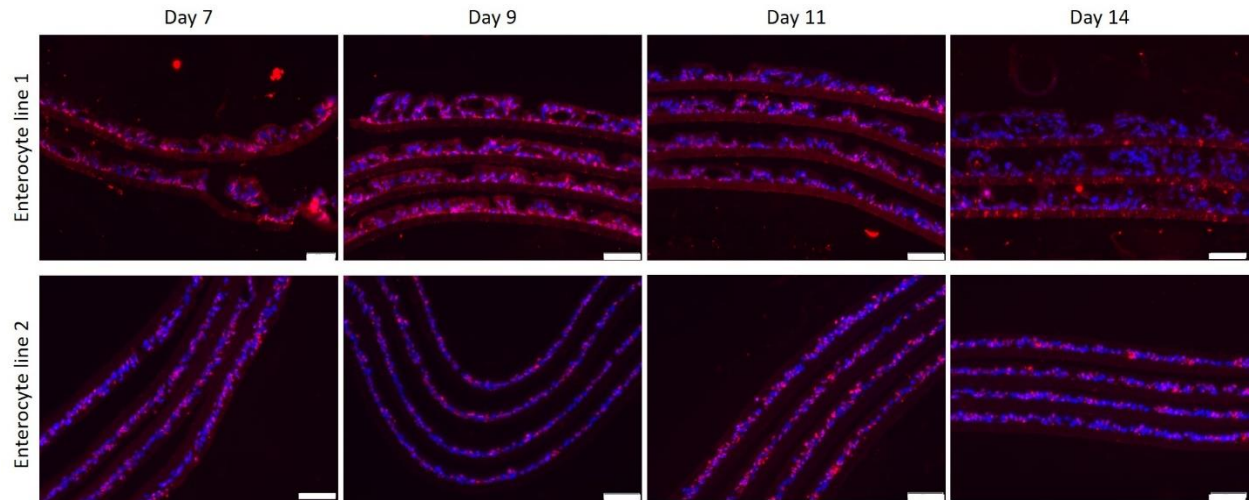
Figure 2-23: TUNEL assay on two different enterocyte lines. Scale bar: 50 µm
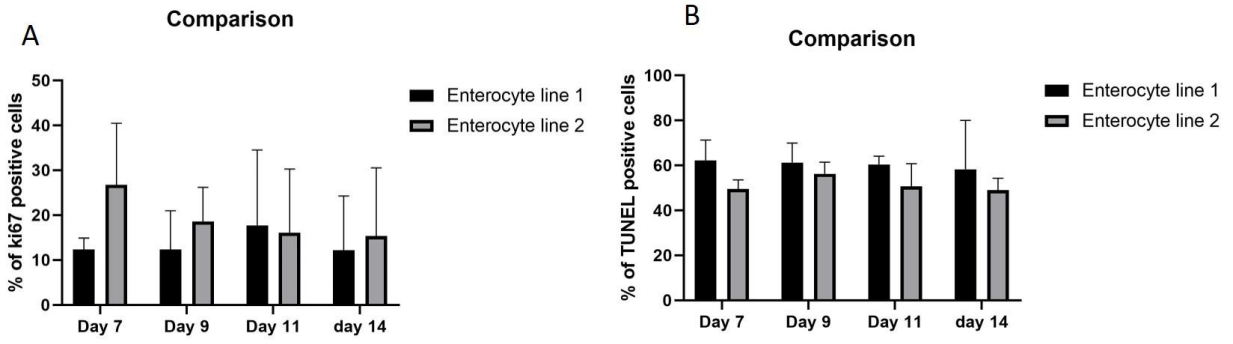
Figure 2-24: Quantitative comparison in Ki67 positive cells and TUNEL positive cells between

two enterocyte lines

No significant difference was observed. (n=3)
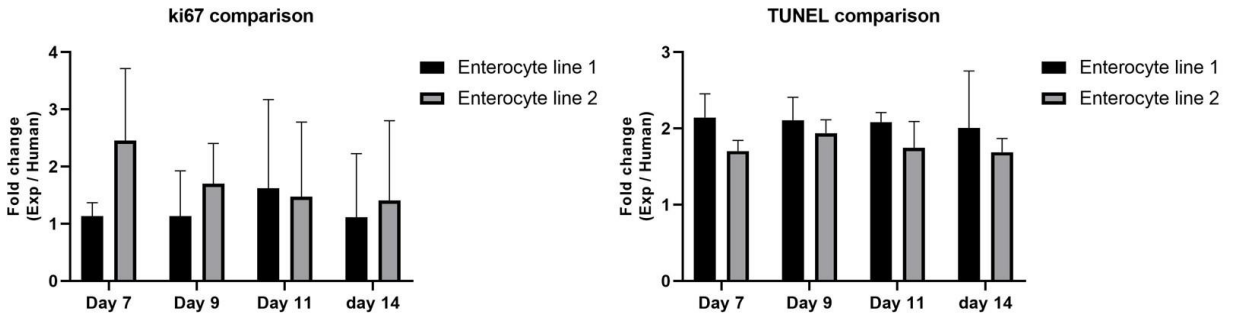
Figure 2-25: Quantitative comparison on ki67/TUNEL between the in vitro cultured monolayers from 2 enterocyte lines and human intestinal epithelium. (n=3)

2.4 Discussion

The most common way to establish a differentiated epithelial monolayer has always been to induce the differentiation via culturing the monolayer with differentiation media (DM). Researchers would first establish the monolayer with proliferation media containing three most important factors which comprise stem cell niche, namely, Wnt3A, R-spondin and Noggin. Then researchers would then switch the media from proliferation media to differentiation media which exclude Wnt3A, r-spondin after the monolayer is established. By culturing with differentiation media, the proliferative cells would then be induced to take on secretive cell lineage, i.e. goblet cells. However, most of the researches conducted with this model would be short-term experiments. The experiments would be conducted for up to 3 days after differentiated monolayer was established. We think this is due to lack of proliferative cell lines in the monolayer to maintain the integrity of the layer once the differentiated cells start going through apoptosis. When comparing the monolayer cultured either with proliferation media or differentiated media for 14 days, it showed that the monolayer cultured with differentiated media indeed was not able to maintain the integrity of the monolayer. This might prove to be a hurdle if one looks to study certain effect on the monolayer in long term.

Understandably, the variabilities among different monolayers are rarely reported since it can only be noticeable when looking at the cross section of the monolayer. The traditional top-down view of the monolayer could not exhibit any observable differences in the monolayer integrity. Additionally, TEER measurement has been widely used to determine the integrity of the monolayer. Experiments or media switch from proliferation media to differentiation media were mostly done when the TEER of the monolayer reached high enough value [19], [22]. It is also often used as parameter to determine the effect of examination factor on the monolayer, such as the effect of drugs on absorption study, the effect of E.Coli or virus on infectious disease study [19]. However, as it can be seen from the H&E results, the variabilities affect the integrity of the

monolayers and subsequently affected the TEER measurement. The variabilities also reflect on the TEER measure which would also pose as an issue that needed to be taken into consideration if TEER is used as an examination parameter of the experiments.

To development a more consistent epithelial monolayer as a study platform, it would be imperative to understand the development of the epithelial monolayer in vitro when cultured with proliferation media as well as the underlying variabilities among different lines that results different morphology since the initial development of the monolayer affects the following development when switching to differentiation media to induce the differentiation. This study focused on one particular line exhibit consistent nonhomogeneous monolayer morphology. Through immunostaining, the monolayer exhibit under-differentiated state without Muc2, l nor localization of villin at the apical region of the monolayer. This under-differentiated state is expected since the monolayer was cultured with proliferation which was used to maintain stemness and inhibit stem cell differentiation at the same time.

Upon examining the proliferative and apoptotic status of the epithelial cells, the percentages of ki67 positive cells are about the same as we would observe in normal human intestinal epithelium, however, the monolayer in vitro exhibit a much higher percentage of apoptotic cells. This imbalance between proliferative cells and apoptotic cells is suspected to be the cause of the disorganized monolayer on the Transwell insert. The strive for balance between proliferation and differentiation on the intestinal epithelium in human body is constant with the cooperation of several different signaling pathways [14], [23], [24]. With limited surface of the Transwell membrane, the balance between the two state would be especially important.

The decrease of the RNA expression level of Lgr5 in enteroids and monolayer indeed indeed is peculiar. However, from the results of re-seed experiment and the examination of Lgr5 expression level in enteroids through several passages, there seems to have Lgr5 positive stem

cells present in the culture. The decrease of the expression level within the passage is suspected to be due to the rapid proliferation of other Lgr5 negative cell types since 3D cultured enteroids is a mixture of Lgr5 positive stem cells and other cell types. As stem cells usually take on asymmetric division [25], the number of stem cells remain relative constant while other cell lineages increase.

In efforts to identifying the variability between two different enterocyte lines which yielded two distinct monolayer morphology, no significant difference could be observed between the two. However, averagely, monolayer derived from enterocyte line 2 possess more ki67 positive cells and less apoptotic cells compare to the monolayer from other line. It is likely that the balance between proliferation and apoptosis was able to be better maintained in the monolayer from enterocyte line 2. The lack of statistical difference was due to high variation among 3 different repeated experiments. One proposed method to test this increase the cellular proliferation with more epidermal growth factor (EGF), which is known to promote cellular proliferation [14], to compensate the high cellular apoptosis observed here.

2.5 Conclusion

Overall, this study highlighted the innate variability exist among different enterocyte lines which is rarely discussed and how it was translated into the development of the epithelial monolayer and the possibility of affect the accuracy of the experimental results based on such platform. We provide evidences suggesting where such variabilities rise from and possible approach to alleviate the variabilities in the monolayer development.

2.6 References

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CHAPTER THREE

THE EFFECT OF AIR-LIQUID INTERFACE (ALI) CULTURING ENVIRONMENT ON THE DEVELOPMENT OF HUMAN INTESTINAL MONOLAYER

3.1 Introduction

Epithelial cells from a cohesive, organized monolayer that lines the interior and exterior of the human body. Some of the organs where it contained empty spaces, such as gastrointestinal track, trachea, lungs, mammary glands and kidney…etc also lines with epithelial monolayers [1]. The epithelial monolayer act as a physical barrier via its tightly packed formation separating the exterior environment with the inside of our body. Though it serves as a barrier, it also selectively allows exchanges of certain ions, hormones, proteins and nutrients in an organ specific fashion [2]. To accomplish this selective function, epithelial cells adopted a certain polarity where each cell is separated into 3 functionally and structurally different domain, namely, apical, lateral and basal domain [2]. Among these three distinct domain, micro tubules, transport vesicles, nucleus, certain transporter proteins would situated at different domain depending on its function [3]. It is a distinct characteristic of the epithelial cells. The signals for the epithelial cells to adopt polarity stems from cell-cell adhesion and cell-extra cellular matrix (ECM) adhesion. Cell-cell adhesion is mediated by e-cadherin to initiate the separation between apical and basal-lateral domain. However, the further distinction of basal domain from others require cell-ECM adhesion which is mediated by integrin. The tight junction protein (i.e. Zo1) is also an useful marker to distinguish different domains. In a fully polarized epithelial cells, the Zo1 would localize at the boundary of apical and lateral domain, whereas in the epithelial cells absent of basal domain, Zo1 would present throughout lateral domain [3]. Morphologically, the polarized epithelial cells exhibit a certain level of plasticity while remain tightly linked to each other through e-cadherin and tight junction protein. The epithelial cells would exhibit morphological alteration in response to the

external stress while maintaining the monolayer integrity. Therefore, the morphology of the polarized epithelial cell can vary, depends on the external stress or the type of epithelial cells. The intestinal epithelial cells in human body exhibit a highly polarized columnar morphology [1]–[6]. However, this columnar morphology is lost in the in vitro cultured intestinal epithelial monolayer.

External stimuli is one of the factor influencing the morphological change of the epithelial cells, several studies started to look at the effect of different in vitro culture environment on the polarization and morphology of the epithelial cells, specifically, tracheal / bronchial epithelial cells [7]–[10]. The alternate culture environment is Air-Liquid interface (ALI) where the epithelial monolayers were only fed basally and the apical side is in direct contact with the air. Under ALI culture, the tracheal epithelial cells reported to be highly polarized and adopt a more columnar morphology compared to traditional culturing condition where the monolayer is submerged under the media. Additionally, the cells under ALI culture were reported to be more differentiated with several differentiated structures presents on the cells, such as cilia and secretory granules [7]–[10]. A. Pezzulo et. al. further reported that the transcriptional profile of several differentiated markers of the ALI cultured epithelial cells showed no significant difference compared to in vivo airway epithelium [10].

Air-Liquid Interface (ALI) culturing method has also since been used on culturing other types of epithelial cells from different animal models. Studies on gastric surface mucus cells from mice and porcine [11]–[13] also showed similar differentiated state and morphological change. The effect of ALI culturing on human gastric mucosa was also explored indicating the similar effect on cellular morphology and differentiation of the epithelial monolayer. Additionally, the increased of mucus production from the ALI cultured monolayer can be seen. The monolayer was further used to investigate mucosal homeostasis and defense against infection [14].

Two methods to maintain murine intestinal organoids has been described ever since the intestinal stem cells have been identified [15], [16]. In the first method, intestinal crypts were isolated before embedding them in Matrigel and culturing in 3D while the other took on a completely different approach [15]. In the second method, the intestinal tissue was minces and embedded into collagen matrix along with epithelial and its stromal cells. The whole tissue embedded collagen matrix was cultured under ALI condition. The study reported that under ALI culturing condition, prolonged cultured could be achieve as well as the presence of sphere-like organoids with proliferation and multilineage differentiation [16]–[19].

Air-Liquid Interface culture has shown to have consistent effect in cellular polarization, morphology and differentiation across several different epithelial cells. However, the effect of ALI culture on human intestinal epithelial monolayer has never been investigated, it would be beneficial if ALI is proven to have the same effect on human intestinal monolayer where it closely recapitulates human intestinal epithelial cell in vivo functionally and structurally.

3.2 Materials and Methods

Isolation of human intestinal crypts

Small intestinal samples were obtained fresh from the Surgical Pathology Department. Tissue was removed from PBS solution and washed multiple times with ice-cold PBS until the solution remained clear. The specimen is placed in a Petri dish containing PBS on ice with the mucosal surface facing upward. Mucosectomy is then performed with surgical scissors and forceps. The mucosa is divided into approximately 1 x 1 cm pieces and washed with cold PBS via vortexing for 30 seconds with three 10-second pulses until the supernatant is clear. These pieces were then incubated in 8 mM EDTA and 1mM DTT solution in PBS for 30 minutes with gentle shaking at 4 ˚C. After the incubation period, the fragments were allowed to settle and the supernatant was discarded. 30 ml of cold PBS is added to the sample, and subsequently vortex for 30 seconds

with 3-second pulses.   15 ml of the supernatant was removed and save on ice. Again 15 ml of PBS were added and the process was repeated six times.   Six fractions were spun down at 100 g for 2 minutes. The supernatant was discarded. The contents of the pellets were examined under light microscopy. Typically, all fractions were pooled together to increase yield of epithelial crypts. The pooled fractions were then purified using a 100 μm and 70 μm pore filters.   The pooled fraction after filtration would be crypts-rich suspended solution.

Maintenance of human intestinal stem cells

Every 250 crypts are suspended in 25 μL Matrigel as described in Sato's 3-D Matrigel culture system developed for murine intestines [15]. The 25 μL of crypt cell/Matrigel suspension is placed in 48-well plate. Matrigel (Fisher) was allowed to polymerize in the incubator for 15   minutes. 250 μL/well of proliferation medium will then be added to the wells. 10 μM Y-27632 dihydrochloride (Fisher) and 2.5 μM CHIR-99021 (Fisher) were added into the proliferation media for the first two days after passage.   The enteroids were maintained in 37°C humidified incubator with 5% carbon dioxide until ideal number of enteroids was reached. The media was changed every two days.   At the time of passaged, the enteroids containing Matrigel buttons were dislodged with P1000 pipettes and along with media, collected into 1.5 mL Eppendorf tubes.   The pellets were acquired via microcentrifuge with three 3-second pulses.   The Matrigel containing pellets were then digested with 500 μL of TrypLE Select disassociation reagent (Thermo Fisher) in 37°C water bath for 5 minutes.   The disassociation reagent was then quenched with DMEM with 10% FBS and 1X AMAB (Life Technologies).   After quenching, the enteroids were further broken down mechanically via pipetting the content with P1000 pipettes repeatedly.   The pellets were then acquired via microcentrifuge with three 3-second pulses.   The pellets were then resuspended with desirable amount of Matrigel and re-seeded into a new tissue plate

Generation of intestinal monolayer and ALI culture

Transswell culturing method was adopted from Julie In et al [20]. The 24-well Transwell inserts (Corning 3414) with polycarbonate membrean were coated with collagen IV (30 μg/mL) prior cell seeding.

The enteroids-containing Matrigel buttons were scraped off from the tissue culture plate with P1000 pipettes and washed with 0.5 mM EDTA/PBS (Sigma). After pelleting the content via centrifuging at 1000 rpm for 5 minutes, the Matrigel/enteroids were then disassociated chemically with 0.25%/0.5mM Trypsin/EDTA in 37 ˚C water bath for 5 minutes. After quenching with DMEM/10% FBS/1X ABAM, P1000 and P200 pipettes were used to break apart the enteroids mechanically, ensuring the enteroids were disassociated into single cells. Cell containing solution were then pass through 40μm cell strainer before centrifuging at 1000 rpm for 5 minutes.

cell pellets were resuspended with proliferation media supplemented with Y-27632 dihydrochloride and CHIR-99021. The cells were seeded into the collagen IV coated Transwell inserts at a seeding density of 750k/cm^2.

The monolayers would be cultured in proliferation media with 100 μL in the insert and 600 μLin the well with media change every 2 days.

 After the monolayer has been cultured with proliferation media 5 days ensuring the monolayer has reached full confluency, Air-Liquid Interface (ALI) culturing condition would be initiated. For the first 5 day of the experiment, the monolayer was cultured with 100 μL of media covered it, the culturing condition was denoted as "Submerged culturing condition". On day 5, to start ALI culturing condition, the media on top of the monolayer would be removed, leaving the monolayer in direct contact to the environment, while the media in the well would be reduced to 350 μL. The media was changed every other day.

Confocal microscopy

The confocal microscopy was done with LSM 880 Inverted Confocal Microscope with AiryScan (Zeiss) at Stanford Cell Science Imagining Facility. The increment of each scan was set at 5 μm.

Cell area and aspect ratio analysis

The cell area and the aspect ratio of the monolayer was measured via ImageJ software. The images of the e-cadherin stained monolayers were used for measurement.

The cell area and the aspect ratio of the human intestinal epithelium was measured via ImageJ software on H&E stained tissue sections.

Images of three randomly chosen area of each section were taken and analyzed. Three different sections / experiments were analyzed (n=3).

Immunohistochemistry

A.   *Sample preparation for histology*

The monolayers were fixed by adding 200 μL of 10% buffered formalin (Fisher) into the inserts for 30 minutes in room temperature. The monolayers were washed with PBS twice after the fixation. The samples needed to be embedded within Histogel (Fisher) in an upright fashion. Liquified the Histogel by warming it up to 65°C and was kept warm for it to remain at liquid state. The polycarbonate membrane was carefully cut off with scalpels. The membrane was them cut into four strips with razor blades and stacked one on top of each other. Place a lid of the 10 cm diameter petridish on an ice block and, with transfer pipette, drops couple droplets of liquified Histogel onto the lid for form a button. Once the Histogel button was solidified due to the drop of the temperature, cut a slit on the button and place the membrane strip into the slit of the Histogel in an upright position so when sectioning, the cross section of the monolayer could be observed. The Histogel button was then covered with fresh Histogel ensuring the strip was covered with Histogel. The button was then leaved on the ice block for 15 minutes before transferring into -20

°C for 10 minutes. Lastly, the strip containing Histogel button was then transferring to the histology cassette and stored in 70% ethanol before submitting to the Stanford Human Pathology/Histology Service Center for paraffin embedding, sectioning and Hematoxylin and Eosin staining (H&E).

B.   *Immunostaining of differentiation marker*

Unstained slides were sectioned at 4 µm thickness with 2 sections per slide. Slides were washed with xylene for 5 minutes twice to remove the paraffin wax. The sections were then rehydrated with progressive decreasing concentration of ethanol in water from 100% to 70% for 2 minutes and finished with 5 minutes incubation in deionized water. Antigen retrieval was done by incubate the slides in 1X Antigen Retrieval Citra Solution (Fisher) for 15 minutes at 100 °C followed by 20 minutes cooling in cold water bath. Samples were then permeabilized with 0.5% Triton-X for 5 minutes and washed with PBS-Tween twice afterwards. A hydrophobic barrier was created with PAP pen (Abcam) before covering the sections with blocking solution containing 2% bovine serum albumin and 5% Normal goat serum for 1 hour in room temperature to prevent non-specific staining. Following blocking, primary antibody working solutions with primary antibody diluted in blocking solution at a ration which is antibody specific was added on top of the designated sections. The sections were incubated at 4 °C overnight. Primary antibodies used would be Muc2 (Santa Curz), E-cadherin (Santa Cruz, abcam), ki67 (Santa Cruz), villin (Santa Cruz). Next day, the slides were washed with PBS-Tween three times to wash away excess primary antibody before adding secondary antibodies which was diluted in PBS-Tween at 1:50 ratio on top of the sections. For the secondary antibodies, Alexafluor 488 goat anti-mouse, Alexafluor 488 goat anti-rabbit, Alexafluor 594 goat anti-mouse or Alexafluor 594 goat anti-rabbit were used depends on the primary antibodies used at 1:200 ratio. Excessive secondary antibodies were washed away with three PBS-Tween wash before adding mounting media with DAPI (Fluoroshield with DAPI,

Sigma) was added. Fluorescent images were taken with Olympus microscope with CellSens software (Olymous).

C.   *TUNEL assay*

The In situ BrdU-Red DNA Fragmentation (TUNEL) assay was performed following the protocol provided by the vendor (ab66110, Abcam) with the exception of 0.5% Triton-X instead of protease K treatment. The samples were counterstained with DAPI at the end.

D.   *Quantification of ki67 and TUNEL*

Immunofluorescent pictures of each samples with ki67 staining and TUNEL assay treatment were taken. Three randomly chosen areas of each slides were taken. Positive ki67 cells were only counted when ki67 and DAPI signals were colocalized. TUNEL positive cells were only counted when its signal was colocalized with DAPI. (n=3 for 3 independent experiments)

E.   ZO1 staining

Samples were fixed at the designated time points with cold Methanol at -20°C for 10 minutes followed by washing with PBS twice. The fixed sample could be stored in PBS or processed for Histology immediately afterwards.

The sample processing protocol prior paraffin embedding, Histology sectioning and H&E staining via Stanford Human Pathology/Histology Service Center had been described previously.

Immunostaining protocol has also been previously described. The samples would be stained against human Zo1 (abcam).

TEER measurement

TEER measurement was done with Epithelial Volt/Ohm meter (World Precision Instrument). Prior the measurement, 250 µL of fresh media was added into the insert and 700 µL of fresh media

was added into the well for all the samples there were either under Normal or ALI culturing condition. STX2 electrode (World Precision Instrument) was sterilized with 70% ethanol followed by washing with the PBS. When measuring, hold STX2 electrode straight up with short end in the insert and long end in the well. The reading on the meter would be in the unit of ohm (Ω). To convert, subtracted the reading with the reading of a blank insert, then multiply by the area of the insert to acquire the unit of Ωcm^2.

After the measurement, the appropriate volume of the media would be adjusted according to its culturing condition.

Differentiation media

The differentiation media was consist of Advanced DMEM/F12 (Invitrogen), 1mM N-Acetylcysteine (Sigma), 2mM GlutaMax-1 (Invitrogen), 1mM HEPES buffer (Invitrogen), 1X B-27 supplement (Thermofisher), 0.5 µM A83-01 (Sigma), 10 nM Gastrin (Sigma), 50 ng/mL recombinant human EGF (Peprotech), 1X PSQ (Thermofisher) and 100 ng/mL Noggin (Peprotech).

L-Lactate analysis

At the designated time points, the supernatants of both culturing condition (i.e. Normal and ALI) were collected, the monolayers were lysed with Lactate Assay Buffer provided by the L-Lactate Asssay Kit (ab65331, abcam).

The lysed cells were treated with Deproteinizing Sample Preparation kit (ab204708, abcam) following the protocol provided by the kit to remove endogenous LDH which would otherwise degrade the lactate.

The L-Lactate determination was performed following the protocol provided by the L-Lactate Asssay kit (ab65331, abcam).

The assay was analyzed via measuring output on a microplate reader at O.D. 450 nm.

ATP analysis

ATP determination was performed following the protocol provided by the Luminescent ATP Detection Assay Kit (ab113849, abcam). The quantity of ATP was determined by converting luminescence measurements measured via microplate reader.

Total protein analysis

Total proteins of the samples were used to normalize the quantity of L-Lactate and ATP. Total protein determination was performed following the protocol provided by Pierce Coomassie (Bradford) Protein Assay Kit (Thermo Fisher).

Analysis of RNA expression

RNA extraction was described in a previous publication [21]. Primer and probe combinations were purchased from Applied Biosystem (Taqman Expression Asssay, Hs00969422_m1(LGR5), Hs00894052_g1(muc2), Hs00167206_m1(SLC11A2), Hs00426232_m1(lyz), Hs00900375_m1(Chga), Hs01031724_m1(vil1), Hs02758991_g1(GAPDH)). RT-PCR was performed in accordance with the description in a previous publication [21] with ABI 7900HT Fast Real Time PCR system. Cycle numbers of all samples were normalized to GAPDH with human intestinal (whole bowel (WB) serving as control tissue.

Statistics

Differences between groups were evaluated via Student's t-test. A p-value < 0.05 was considered as statistically significant.

3.3 Result

The effect of ALI on monolayer morphology

The ALI condition was initiated on Day 5 after seeding. After 2 days under ALI condition, the monolayer started showing signs of morphological change (Fig. 3-1). The thickness of the cell started to increase. Epithelial cells became columnar shape which more closely resemble the enterocytes of human intestine. Significant difference in the cellular morphology can be observed. Although monolayer showed signs of polarization where nuclei shifted to the basolateral side at early time point (i.e. day 7) when cultured under submerged condition, it gradually lost its polarity and integrity while remaining relatively flat when compare to the monolayer cultured under ALI condition.

The effect of ALI on the monolayer generate from enterocyte line 2 exhibited the same morphological change under ALI condition (Fig. 3-2). The monolayer thickened and took on a columnar shape. It demonstrated that ALI has the same effect among monolayers generated from different enterocyte lines. One thing to note here is that the differences between the two lines was not lost. The monolayer generated from enterocyte line 2 was still extremely organized even under ALI condition.

With confocal microscopy, the entire depth of the epithelial monolayer was scanned through from apical to basal side of the monolayer (Fig. 3-3). When comparing the apical side of the monolayer, the presence of the microvilli (the green dots presented in the within the outline of each cells) could be seen whereas the microvilli was absent on the monolayer under submerged condition.

Furthermore, when scanning toward the basal side of the monolayer, the difference in the morphology and the organization can also been observed (Fig. 3-3). The monolayer cultured under ALI exhibited a much more organized, distinctive cobblestone pattern throughout the apical-

Figure 3-1: H&E histology of the monolayer derived from enteriocyte line 1 cultured in two different conditions. Scale bar: 50 μm

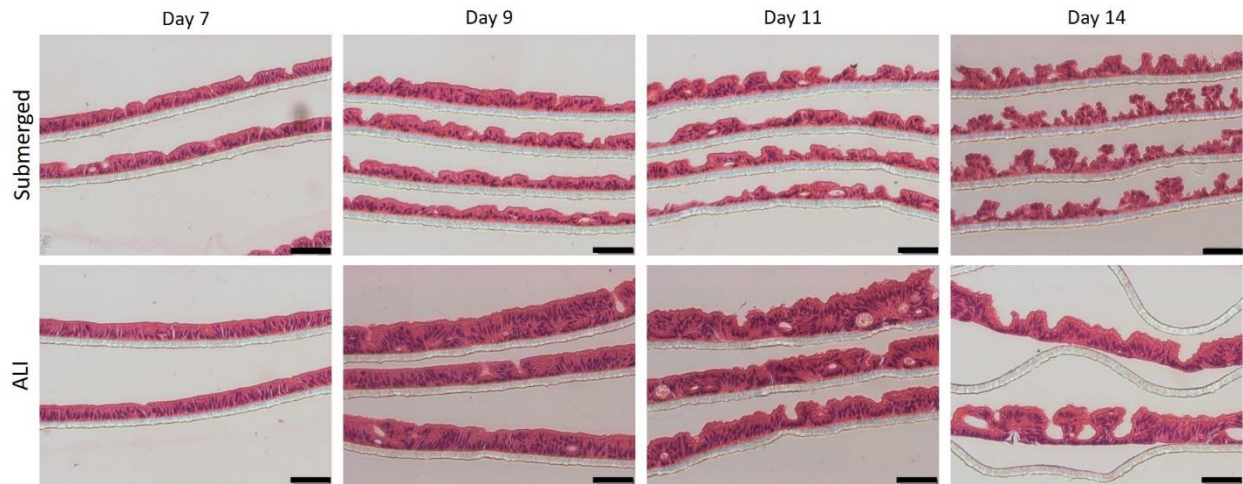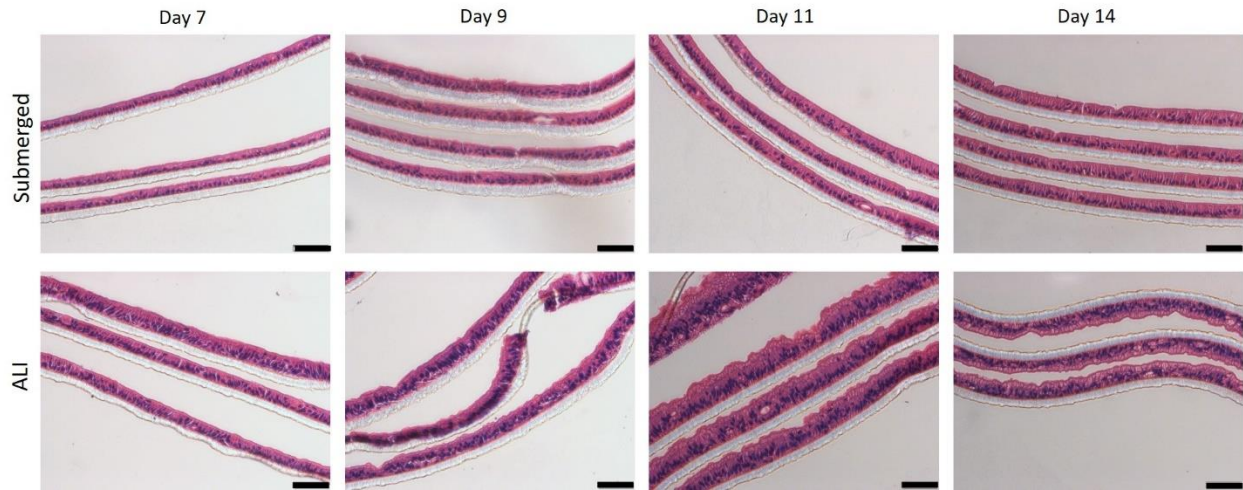Figure 3-2: H&E histology of the monolayer derived from enterocyte line 2 cultured in two different conditions. Scale bar: 50 μm
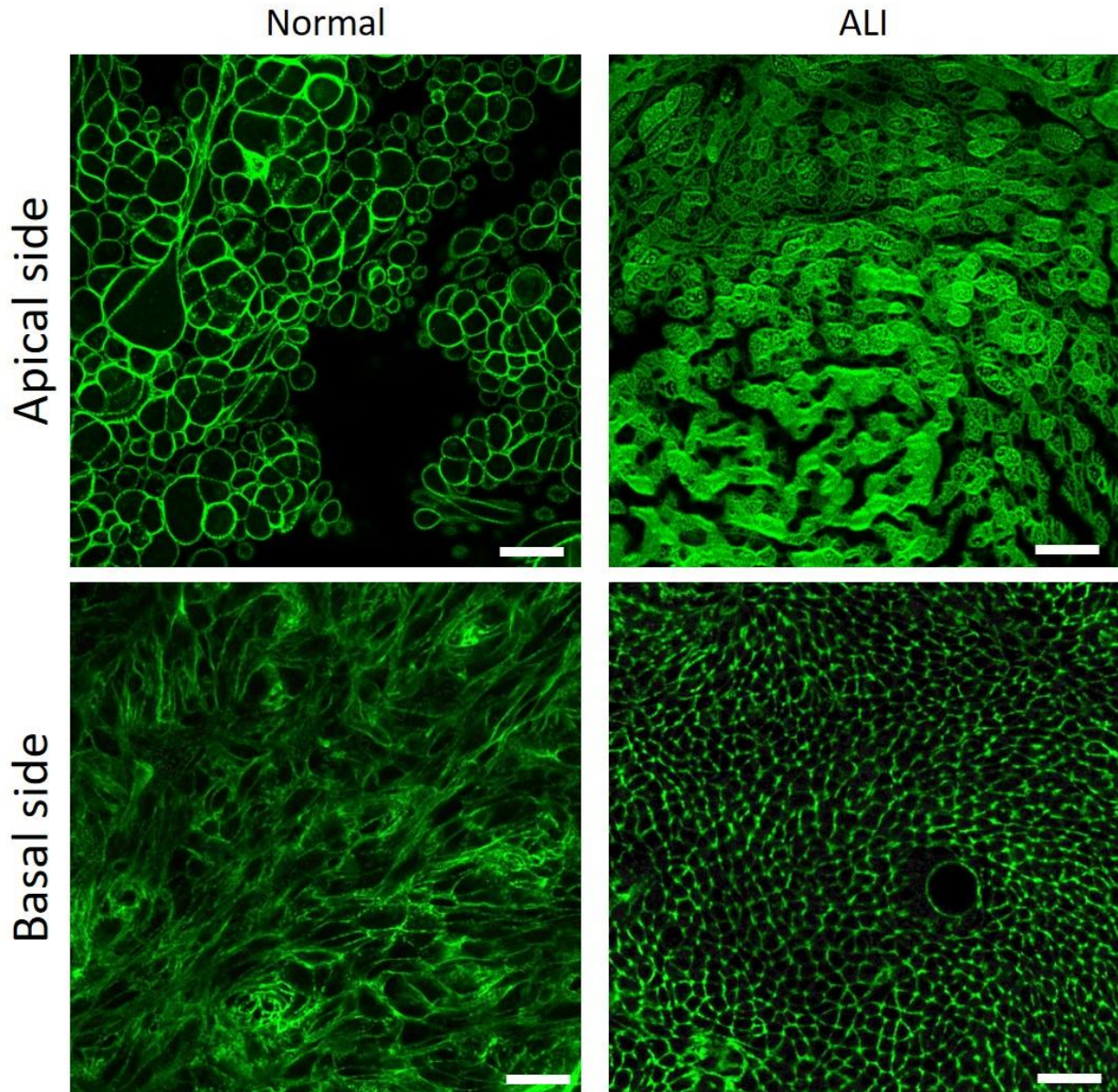
Figure 3-3: confocal microscopy images of the monolayer under two different culturing conditions from apical to basal side. Apical side of the monolayer under ALI exhibited the presence of micro villi and exhibit organized cobble stone pattern at the basal side. Scale bar: 25 μm

basal axis than the one cultured under submerged condition where it started to lose the integrity close to the basal side of the monolayer.

The result suggested that ALI had positive effect on monolayer development.

With examination on f-actin staining via confocal microscopy on the monolayer cultured under ALI condition, the f-actin localized at the apical side of the monolayer confirmed the polarization of the epithelial cells and the presence of actin-rich brush border (Fig. 3-4).

Morphology characterization of the epithelial cells

To assess whether the thickening of the monolayer is due to enterocyte itself being elongated and take on a columnar shape or simply due to increase in cell number, we examined the cross-sectional area and its aspect ratio of the single cells under submerged and ALI condition (Fig. 3-5). When compare the area of the cells under two conditions, it showed that they have similar cross-sectional area where the area ratio of the two remained around 1 from day 7 to day 14. However, when compare the aspect ratio of the cells under these two conditions, the aspect ratio of the cells under ALI condition was significantly higher than its counterparts under submerged condition. The result demonstrated that the increased thickness of the monolayer under ALI condition is indeed due to the cells took on the columnar shape.

To further examine the morphological similarity of the monolayer and the epithelium of the human intestine, we employed the similar strategy comparing the cross-sectional area ratio as well as the aspect ratio of the two (Fig. 3-6). The cell-area ratio was derived by comparing the cell area of the monolayer and the cell area of the histology sections of the human intestine. The result showed that the cross-sectional area of the cells from the monolayer under both submerged and ALI condition shared similar area with enterocyte of the human intestine. On the other hand, when comparing the aspect ratio of the cells of the monolayer and human intestinal epithelium, the cells of the monolayer under ALI condition took on a more similar morphology than the cells

70
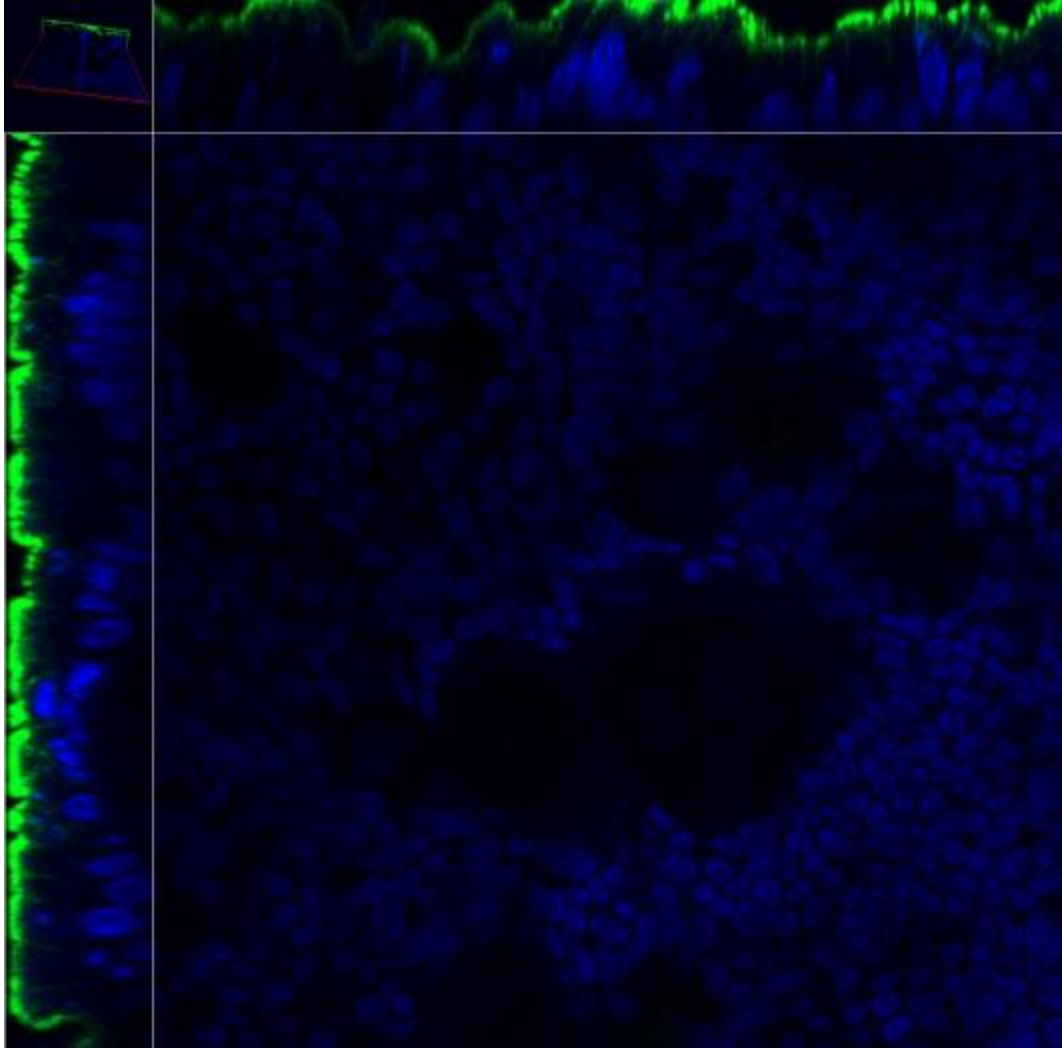
Figure 3-4: confocal microscopy images of the monolayer stained with FITC Phalloidin against

F-actin. The cross-section of the monolayer showed actin-rich brush border present at the

apical side of the monolayer. Scale bar: 25 μm
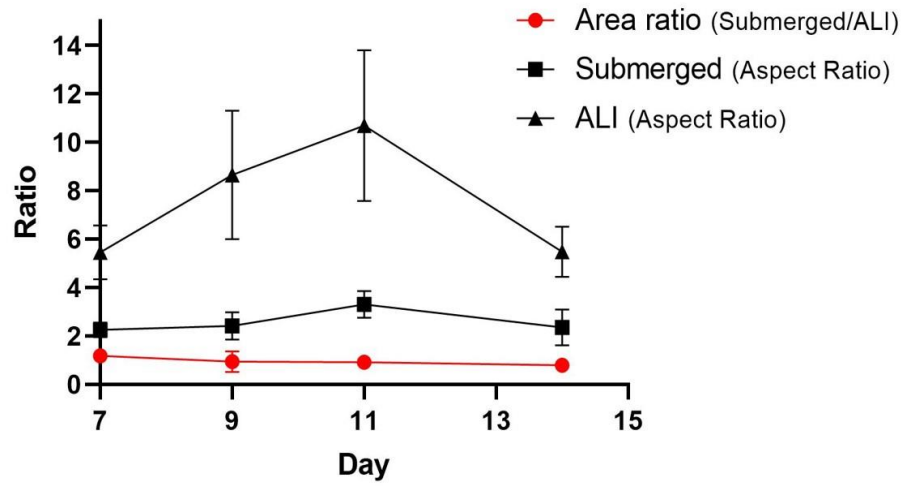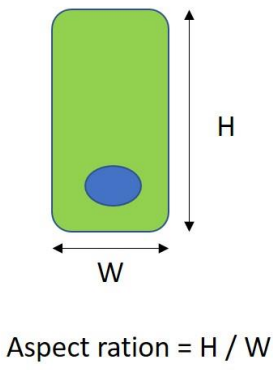
Figure 3-5: Cross section area and aspect ratio analysis of the cells. There was significant difference in the aspect ratio of the cells between Normal and ALI yet had similar cross section area. Aspect ratio = H/W. (n=3)

A cell area comparison (w/ Human tissue)
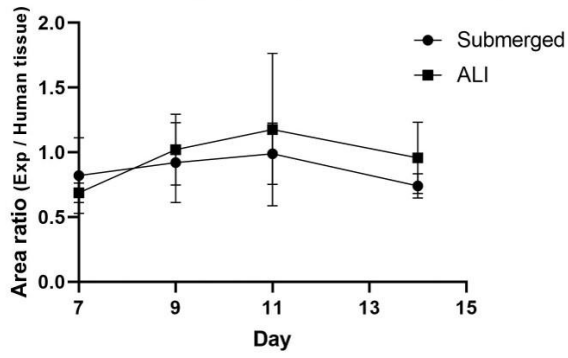
B Cellular aspect ratio comparison (w/ Human tissue)
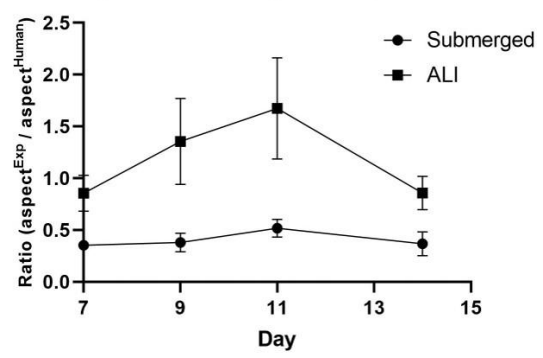
Figure 3-6: The comparison of cross section area (A) and aspect ratio (B) of the cells between in vitro monolayer and human intestinal epithelium. Aspect ratio = H/W. (n=3)

under submerged condition. This result suggested that not only the cells under ALI condition underwent morphological change, their morphology is closely resembled the morphology of the enterocyte of the human intestine.

Functional analysis on the monolayer

Tight junction development is an essential characteristic for an epithelial layer which possess the integrity to function as a barrier. Loss of tight junction would result in a permeable epithelium with which various pathogens and toxins could pass through easily.

From the tight junction protein, Zonula occludens-1 (Zo1), staining (Fig. 3-7), the localization of Zo1 at the apical side of the monolayer can be seen up to day 14 when cultured under ALI whereas there was little to no Zo1 expression on the monolayer cultured under submerged condition at day 14.

The result indicated that the monolayer lost its integrity at the later time point when cultured with media on top.

The difference in the tight junction development on the monolayer between two culturing conditions was also reflected on TEER value measurement (Fig. 3-8).

TEER value was measured on the monolayers cultured under two culturing conditions from day 1 to day 14. After ALI was initiated on day 5, TEER value bifurcated. The TEER value of the monolayer under submerged culturing condition kept increasing until the sharp decrease on day 14 whereas TEER value of ALI cultured monolayer remained at a relatively stable level around 100 Ωcm^2 throughout the experiment.

Although submerged cultured monolayer exhibit higher TEER values, it also showed higher variabilities compared to the monolayer under ALI culturing. Furthermore, studies [22] have

Figure 3-7: Zo1 (red) staining on the monolayers cultured under Submerged and ALI conditions.

Scale bar: 50 µm
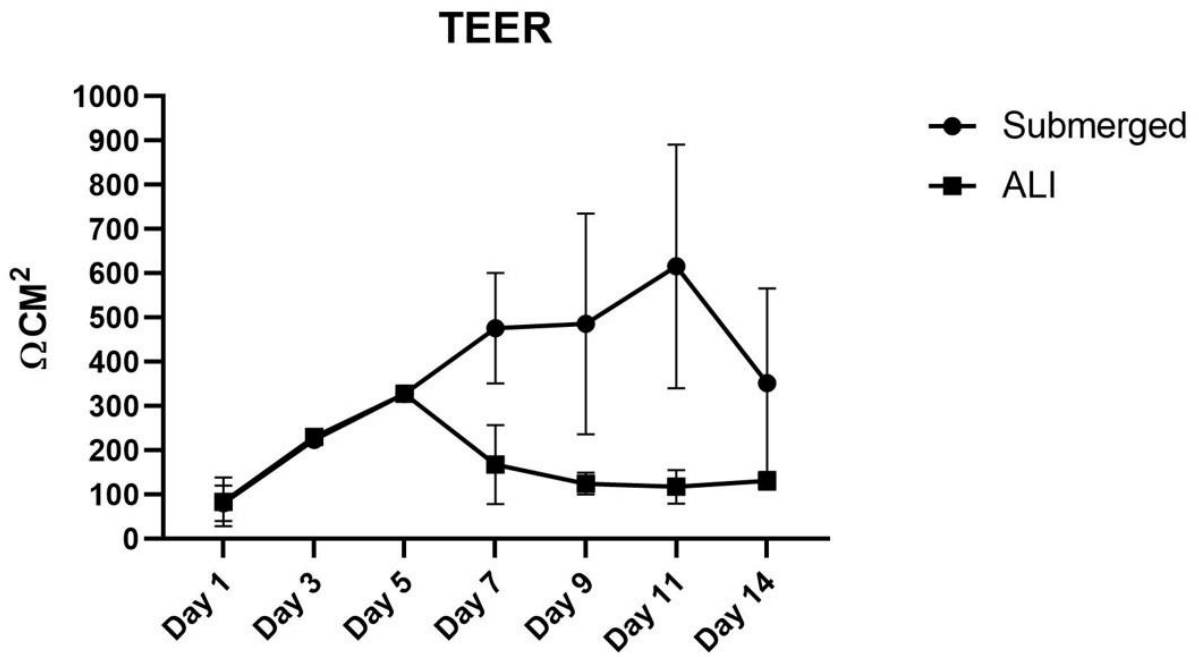
Figure 3-8: The measurement of TEER value on monolayers cultured under two conditions.
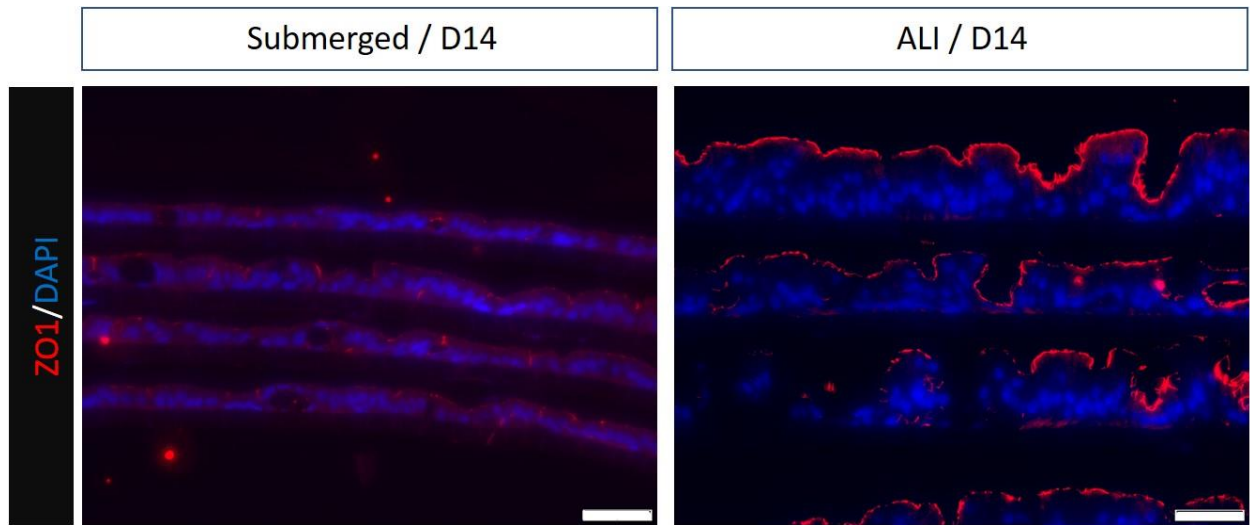
Significant difference can be seen after the ALI was initiated. (n=3)

suggested that high TEER value was not physiologically relevant. The TEER value of the human intestine tissue was around 100 Ωcm^2.

Ki67 / TUNAL analysis

Proliferative cells (ki67 positive) could be seen on the monolayer cultured under two conditions throughout the experiment (Fig. 3-9).

Upon examining the cellular apoptosis via TUNEL assay, the cells cultured under ALI condition seems to experience less apoptotic event compared to the cells under submerged culture condition (Fig. 3-10).

A more quantitative approach was employed examining the difference in the proliferative cells and apoptotic cells between the two monolayers (Fig. 3-11). The results showed that there is no significant difference in the percentage of the ki67 positive cells. However, it showed significant difference in the percentage of the apoptotic cells. There were generally less cells underwent apoptotic events when culturing under ALI condition, especially at day 11. It is likely that because there was less cell death, the monolayer under ALI conditions seems to be able to maintain longer and better than the one under normal condition.

Further comparing proliferative and apoptotic cells between monolayer in vitro and human intestinal epithelium indicated that the percentage of the cells underwent apoptotic event was similar when monolayer was cultured under ALI condition (Fig. 3-12).

Immunostaining of the monolayer

A panel of antibodies were used to examine the effect of ALI on the cellular differentiation. Under the ALI condition, no Muc2 positive cells were observed (Fig. 3-13).

From the villin staining, differences were observed between two culturing conditions (Fig. 3-14). Villin signals localized at the apical side of the monolayer while there was no colocalization at the

Figure 3-9: Immunofluorescence of cultured epithelial monolayer from day 7 to day 14 under two culture condition. Ki67 positive cells could be seen in both culturing conditions. Scale bar: 50 µm
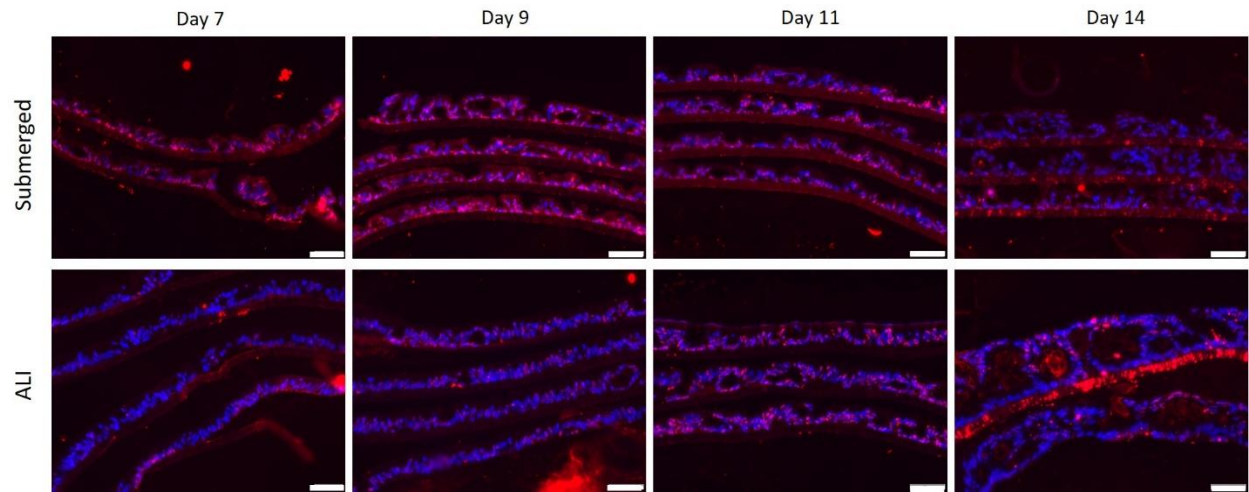
Figure 3-10: TUNEL assay on the monolayers culturing under two culturing conditions. Scale bar: 50 μm
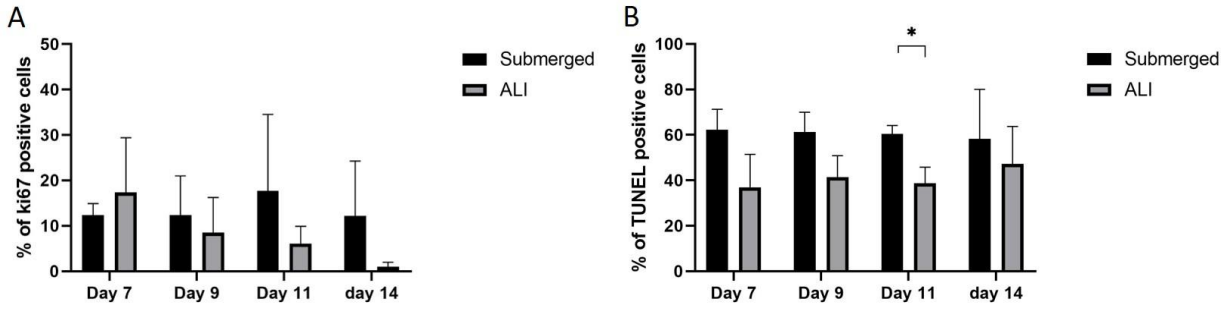
Figure 3-11: Quantitative analysis of Ki67 positive cells and TUNEL positive cells between two culturing conditions.

No significant difference was observed in the percentage of ki67 positive cells. Lower cellular apoptosis in the cells with ALI condition. (n=3). (* p < 0.05)

ki67 comparison
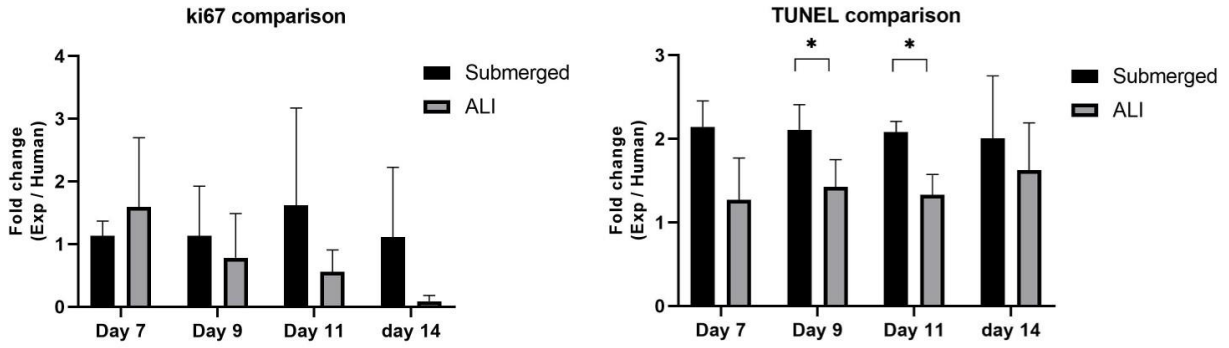
TUNEL comparison

Figure 3-12: Quantitative comparison of Ki67 positive cells and TUNEL positive cells between in vitro cultured monolayer and human intestinal epithelium.

No significant difference was observed in the comparison of ki67 positive cells. Under ALI culturing condition, the percentage of apoptotic cells decreased to similar level as on human intestinal epithelium. (n=3). (* $p < 0.05$)
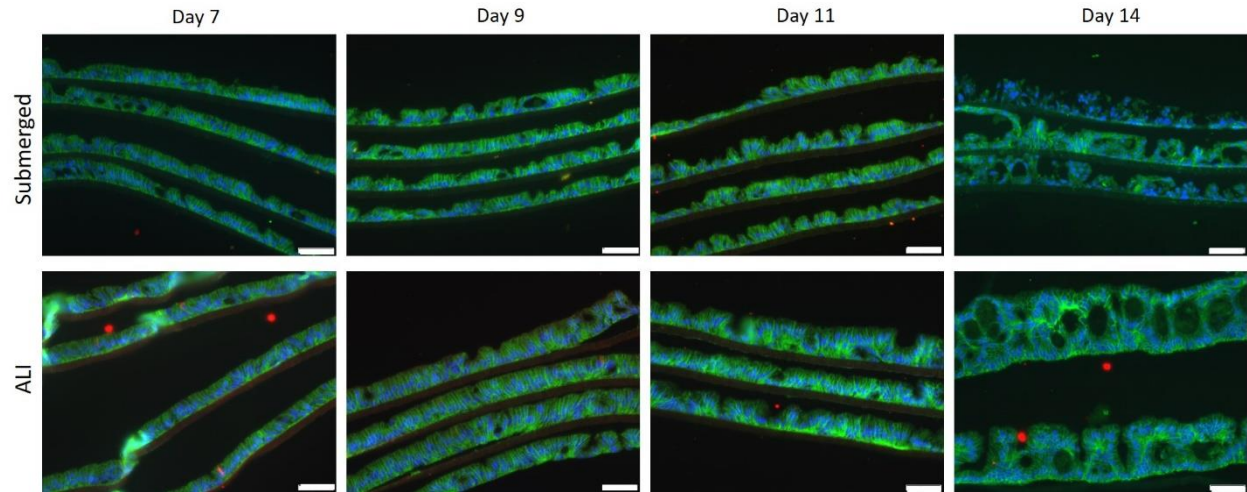
Figure 3-13: Immunofluorescence of cultured epithelial monolayer from day 7 to day 14 under two culture conditions (E-cadherin: Green, Muc2: Red, DAPI: Blue). Both monolayer lacked Muc2, a marker for goblet cells.
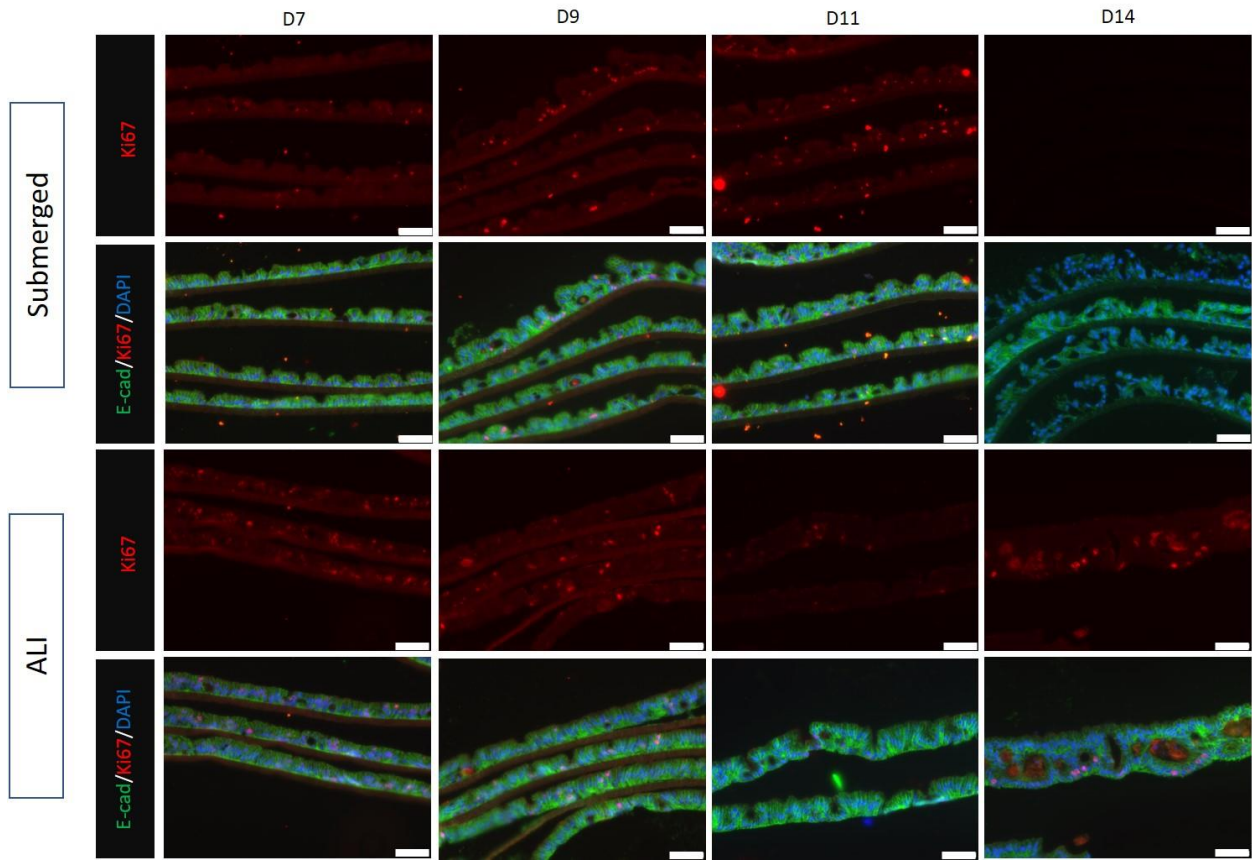
Scale bar: 50 µm
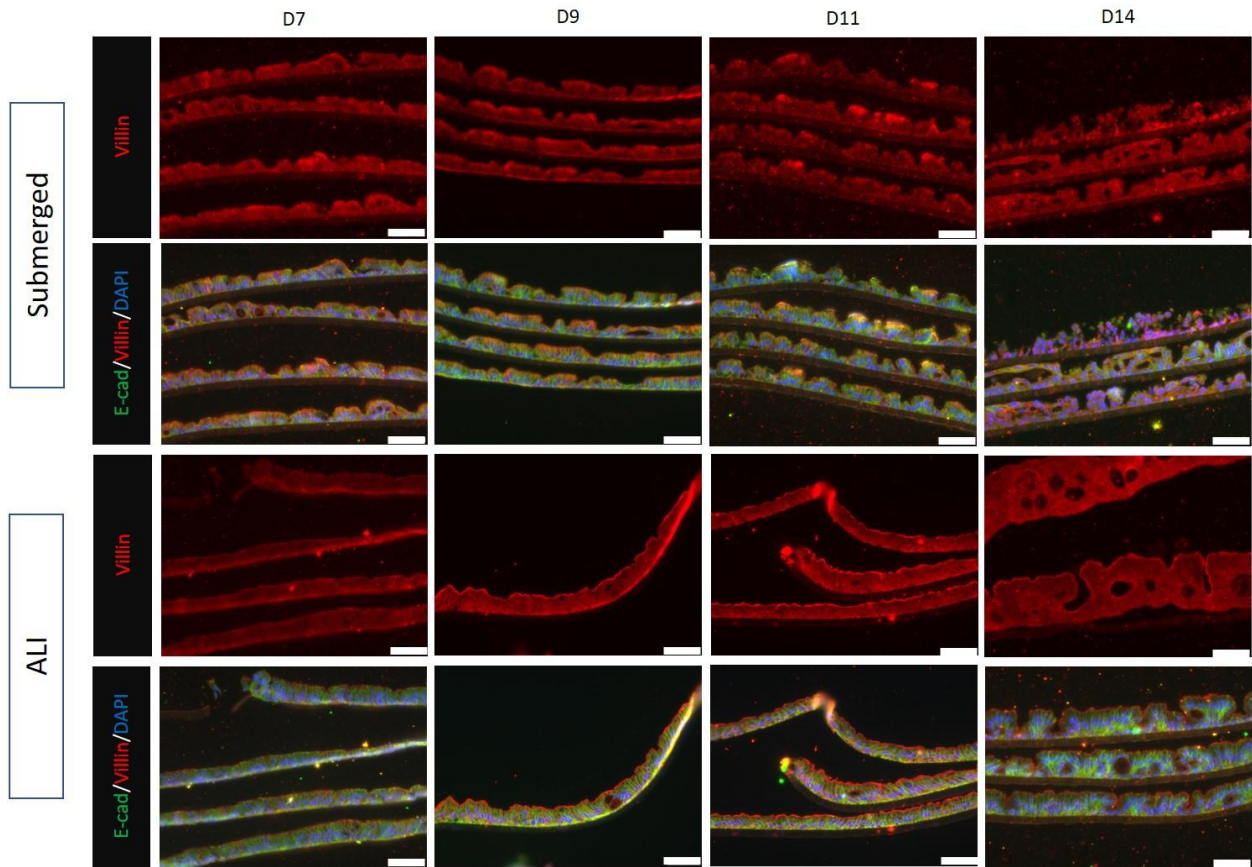
Figure 3-14: Immunofluorescence of cultured epithelial monolayer from day 7 to day 14 under two culture conditions. Villin signal can been seen localized at the apical side of the monolayer under ALI condition. Scale bar: 50 μm

apical side of the monolayer under submerged condition. The localization of the villin at the apical side of the monolayer indicating the presence of brush border composed of microvilli at the apical region of the epithelial cells. In human intestine, it is only present on the polarized, differentiated enterocyte. This result suggested that even though there no secretory cell lineage present (i.e. Goble cell), the monolayer under ALI was still at a more differentiated state compared to the one under submerged condition.

Analysis of RNA expression

The RNA expression of the various differentiation markers was examined via quantitative RT-PCR (Fig. 3-15). Both MUC2 and CHGA showed significant low expression overall. However, lysozyme, villin and SLC11a2 had relatively high mRNA expression. Although some genes show little to no expression, the differences between submerged and ALI culturing condition could still be observed. Across this panel of genes, the expression under ALI condition consistently had significantly higher expression than the one under submerged conditions. This difference in gene expression again indicate that ALI has a positive effect on cellular differentiation. However, the effect the ALI has on the monolayer was not the same as differentiation media where it induces differentiation through transforming proliferative cell lineage to secretory cell lineage. The effect of the ALI on cellular differentiation seems to be heightening the existing differentiation cue. Therefore, a relatively higher expression of lysozyme and villin than human intestine was observed since the expression of these two genes were already existed under normal culturing condition. On the other hand, Muc2 and CHGA genes were not expressed under normal culturing condition, hence even though the gene expression under ALI condition was still heightened, the overall relative expression still remained low.

The effect of ALI on monolayer differentiation while providing differentiation cue
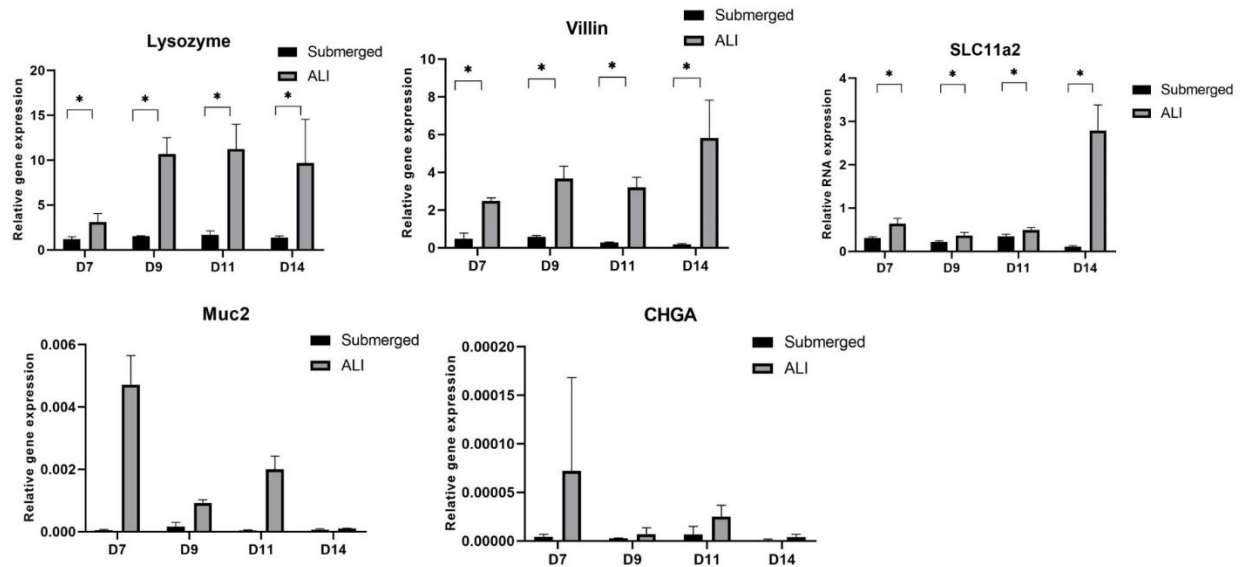
Figure 3-15: RT-qPCR analysis on multiple differentiation marker for the epithelial monolayers. The expression of Lysozyme, Villin and SLC11a2 showed significantly higher expression in the monolayer under ALI condition. Other two marker (Muc2, CHGA) also showed the same trend, however, overall expression remained low). Human intestine was used as control. (n=3). (* $p < 0.05$)

To further confirm the unique effect of ALI on the monolayer development where it only heightens the existing differentiation cue, we examined the effect of combining differentiation media and ALI at the same time on the monolayer.

When examining the effect on the cellular differentiation with Muc2 (Fig. 3-16), there was significantly more Muc2 positive cells in ALI condition. In this experiment, the differentiation cue was provided by switching culturing media from proliferation media to differentiation media. Therefore, several Muc2 positive cells were observed under submerged culturing condition.

Analysis on the difference in metabolic mechanism

Studies conducted on cow epithelium have suggested that the effect of ALI was due to cells under different culturing conditions take on different metabolic mechanisms [7]. The metabolic mechanisms of the cells under submerged culturing condition lean more toward glycolysis due to limited oxygen supply via transfusion through a layer of media on top of the monolayer whereas the monolayer under ALI condition is in direct contact with the oxygen, hence exhibit increased oxidative mechanism. Two main differences between two metabolic strategies are ATP and L-Lactate production. Glycolysis would yield less ATP production and higher L-Lactate production. To examine if this is the underlying reason to the effect in ALI condition, we look at the production of these two (Fig. 3-17). Both L-Lactate production and ATP production were all normalized to total protein. The result showed that there is no significant difference in the ATP production and the accumulated L-Lactate in the supernatant between the two culturing conditions, however, there is significant difference in L-Lactate production within the cells. The cells under ALI condition produce less L-Lactate than the ones under submerged condition consistently throughout the experiment. Although there was no difference in ATP productions, L-Lactate production within the cells still showed significant difference which suggest that the differences in the metabolic strategy plays a role but might not be a key factor as reported.
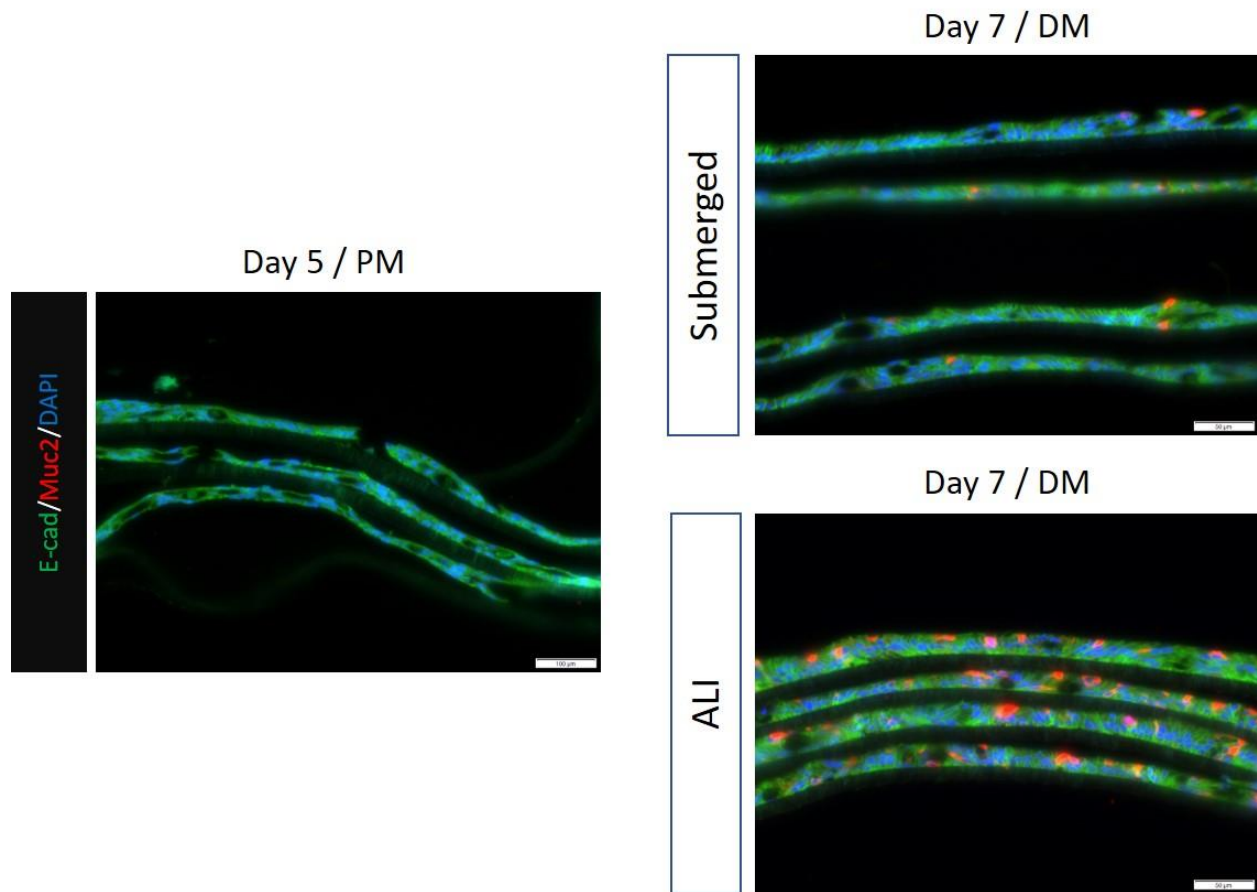
Figure 3-16: Immunofluorescence of Muc2 expression on the monolayer on day 5 and day 7 cultured with differentiation media (DM). The media was switch on day 5 from proliferation media (PM) to differentiation media. ALI was initiated at the same time. The Muc2 expression was much higher in the monolayer cultured under ALI condition.
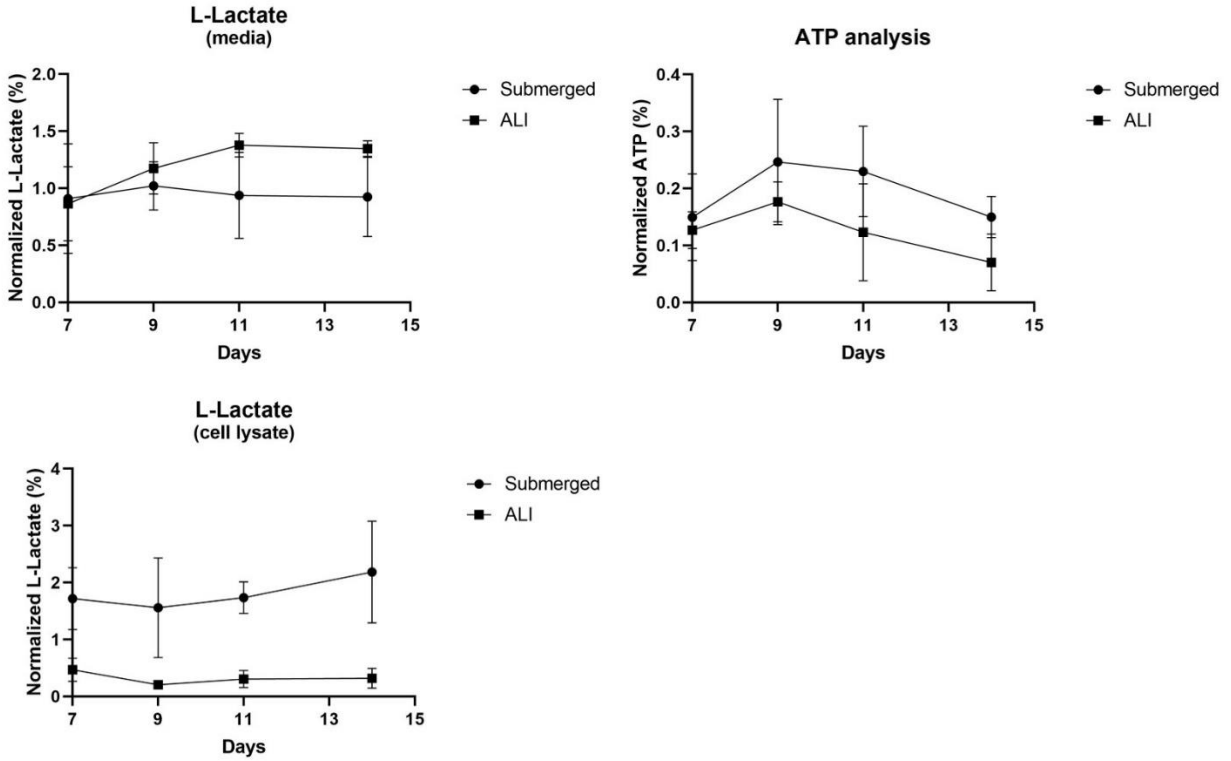
Figure 3-17: Quantitative analysis on the L-Lactate and ATP production. A significant difference

of L-Lactate production can be observed between the cells cultured under Submerged and ALI

conditions.

No significant difference in ATP production between the two.

Results were normalized to total protein. (n=3)

3.4 Discussion

Air-Liquid Interface (ALI) culturing condition has been widely in tracheal epithelial cell culture. Through ALI culturing condition, the tracheal epithelial cells became more differentiated with cilia formation [7]–[10]. This study demonstrated that ALI culturing condition has the similar effect on intestinal epithelial cell. When intestinal epithelial cells were cultured under ALI condition, the cells adopted a more columnar morphology resembling the human enterocytes as well as formation of brush border at the apical region in vitro. Furthermore, our data suggest that ALI also has a positive effect on the maintenance of the monolayer integrity, not only the tight junction was able be maintained for up to 14 days, the TEER value remained stable at a physiological relevant range once ALI had been initiated. One of the reasons to the positive effect on monolayer maintenance is suspected to be the decrease in the apoptotic cells. The percentage of apoptotic cells was comparable to the ones in human intestine.

Although no secretory cell lineage (i.e. goblet cell) could be seen on the ALI cultured monolayer, immunostaining results indicating the formation of brush border and ferroprotin iron transporter at the apical region of the monolayer. This result combining the quantitative RNA expression analysis via RT-PCR suggested that the monolayer cultured under ALI condition exhibited a more differentiated state than the one cultured under submerged condition.

Admittedly, several secretory cell lineage gene expressions (i.e. Muc2, CHGA) remain at a relatively low range, this expression is likely due to the fact that this monolayer was still cultured with proliferation media which inhibit cellular differentiation. However, this observation and the results from RNA analysis provided another interesting insight as to the role ALI culturing condition has on monolayer development. Although several secretory cell lineage gene expressions (i.e. Muc2, CHGA) remain at a relatively low range, the ALI cultured monolayer consistently exhibiting higher expression level compare to the one under submerged culture. This

likely suggested that ALI culturing condition act like an amplifier, it can only amplify the existing differentiation cue but could not direct the cellular differentiation toward different cellular lineage.

To confirm this hypothesis, we decided to provide the differentiation cue via switching the media from proliferation media to differentiation media. The result indeed demonstrated that there were more Muc2 positive cells in the ALI cultured monolayer.

Several studies have been conducted in efforts to understand the underlying effect of ALI on the monolayer development [7], [13]. Studies suggested that the difference between ALI culture and submerged culture stems from different metabolic mechanism being utilized. With ALI culture, there was direct oxygen supply, the cells demonstrated increased oxidative metabolism. On the other hand, the cells that were submerged cultured leans toward glycolysis [7]. To investigate if this difference was also present in our two culturing systems, we examined the L-Lactate and ATP production where there would be more ATP production and less L-Lactate production with oxidative metabolism. Although there were no significant differences in ATP production and L-Lactate in the supernatant, there is significant difference in the L-Lactate production within the cells. This result indeed suggested that there seems to have increased oxidative metabolism when ALI cultured, the difference in the different metabolic mechanism is not likely a deciding factor. Further investigating is required.

One study done by Yokoyama et. al. [12] suggested that mitogen-activated protein kinase (MAPK) pathway was activated under ALI culturing condition. They observed the increase in phosphorylated ERK-1 and ERK-2 via western blot. Furthermore, after treating the ALI cultured cells with MAPK inhibitor, U0126, the morphological change was not present. MAPK pathway is known to regulate cellular proliferation, differentiation, actin cytoskeleton reorganization and apoptosis [23], [24]. It would be an interesting step to examine the MAPK activities in our culturing system.

Another study from Altay et. al. [25] achieved similar monolayer morphology on mice intestinal epithelial cells via culturing the monolayer with two different media at the same time. The media in the well would be nutrient rich compare to the media in the Transwell. The nutrient gradient achieved through this method could also achieved via ALI culturing condition since the media would be absent in the Transwell. With the fact that the thickness of the monolayer in this study also increase, one cannot help but wonder whether the key factor contributing the effect of Air-Liquid Interface culturing condition on the monolayer development is the presence of the nutrient gradient across apical-basal axis. This would be an interesting next step to this study in discovering the underlying driving force of ALI culture.

3.5 Conclusion

This study demonstrated the positive effect of Air-Liquid Interface culturing on the monolayer development. Under ALI, the monolayer adopted a more physiological relevant morphology. It still possesses proliferative capability with decrease apoptotic cells. Furthermore, the ALI cultured monolayer is more differentiated compare to its counterpart under submerged culture. Lastly, it is believed that ALI culturing condition serves more as an amplifier where it could enhance the existing differentiation cue, but not directing the cellular differentiation. ALI culturing condition provide us with a more physiologically relevant human intestinal epithelial monolayer in vitro in both morphological and differentiation perspective.

3.6 References

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CHAPTER FOUR

THE INTERACTION BETWEEN INTESTINAL EPITHELIAL MONOLAYER DEVELOPMENT
AND INTESTINAL SUBEPITHELIAL MYOFOBORBLAST (ISEMF)

4.1 Introduction

To maintain the homeostasis of the stem cells between proliferation and differentiation, the term,
stem cell niche, was brought up in late seventies [1]. Stem cell niche is described as a
microenvironment where the stem cells were situated and it maintained the homeostasis of the
stem cells through several different pathways and paracrine signals [1]–[5]. The intestinal stem
cell niched has been used to describe various strategies and players involved in maintaining the
homeostasis of the intestinal stem cells in the molecular and cellular level [1]–[3]. It is believed
that the stem cell niches is comprised with contacting epithelial cells and other non-epithelial cells
situated in close proximity to the intestinal stem cells [3]. There are several signaling pathways
involved in the homeostasis of the intestinal stem cells (ISCs), among all, canonical wnt signaling
pathway and BMP signaling pathway are two balancing forces of the stem cell proliferation and
differentiation [2]. BMP signaling is most active in the villius area of the cryp-villus axis where all
the differentiated intestinal epithelial cells situated and is inhibited in the crypt area where all the
cellular proliferations take place [2]. On the other hand, wnt signaling has been known to be an
essential signaling pathway to the maintenance of the ISCs. Each signaling pathway required
certain protein or molecules to active or inhibit the activities of the pathway.

For the wnt signaling pathway, when the receptor on the stem cell membrane engaged with Wnt
factors, it will stabilize β-catenin and translocate to the nucleus to activate the transcription factors
which results in the maintenance of the ISCs. Additionally, R-spondin is known to be an agonist
of wnt signaling which enhances the activities of the pathway [4], [6]. Due to the success of the
in vitro culture technique of the ISCs [6], it has been suggested that several of these growth factors

comes from the neighboring Paneth cells where it situated next to the stem cells in the intestinal crypts. Paneth cells has been proved to be an exogeneous source of these growth factor in vitro via and experiment conducted by Sato et. at. [4] where through co-culture with Paneth cells, single Lgr5 positive stem cells were able to be maintained in longer term compare to mono-culture.

Despite the in vitro experimental results indicating the important role the Paneth cells play in the maintenance of the ISCs, experiments done in vivo suggested otherwise [5]. Unexpectedly, when Wnt3 was conditionally deleted in the mice model, the proliferation of the stem cells was not affected. Additionally, the maintenance of the stem cells was also undisturbed in secretory cells lacking mutant mice. These finding suggested that there is another source of Wnt signals from the nonepithelial cells which serves as a redundancy in maintaining the survival of the intestinal stem cells.

Non-epithelial stromal cells in close proximity of the intestinal stem cells also have been shown to be part of the stem cell niche where they secrete various paracrine signals contributing the maintenance of the homeostasis of the ISCs [5], [7], [8]. Among various stromal cells involved in the stem cell niche, intestinal subepithelial myofibroblast (ISEMF) has been recognized playing an important role in stem cell maintenance [8]. Intestinal subepithelial myofibroblast (ISEMF) is α-smooth muscle actin positive stromal cells situated in submucosal layer, in close proximity to both crypts and villus region. ISEMFs share phenotypic characteristics of both fibroblast and smooth muscle cells. These cells mediate information to and from the intestinal epithelium through secreting various cytokines, growth factors, chemokines…etc. Through this, they play an important role in intestinal organogenesis [9]. To maintain the homeostasis of the intestinal epithelium, two perspectives need to be considered and work in balance, namely, proliferation and differentiation. It has been suggested that ISEMFs take part in both of these developmental events [10].

Several studies have been conducted to investigate the role ISEMFs play in both proliferation and differentiation of intestinal epithelial cells [11]–[16]. Lahar et. al. first showed that through seeding the freshly isolated intestinal crypts on top of a confluent feeder layer of ISEMFs, the subsequently formed enteroids were larger and were able to be maintained for up to 56 days compared to only 6 days when mono-cultured. Additionally, through co-culture, it eliminated the need for the exogenous Wnt3a and R-spondin [11]. Jabaji et. al. further showed that, instead of physically co-cultured with ISEMF, the ISEMF conditioned media was also viable and have the same effect on enteroid development [12]. Interestingly, through RT-PCR analysis, the expression of both stem cell and differential marker were both presented. Further analysis on ISEMF via RNA sequencing done by Lei et. al. revealed that several genes coding for secreted proteins of the Wnt pathway were upregulated with at least two-fold difference. One particular gene was R-spondin 2, which is a agonist of the Wnt signaling pathway [13].

Through the experiments described above, it is apparent that ISEMF plays an important role in stem cell maintenance. However, 3D cultured intestinal enteroids used in the experiments did not accurately reflect the anatomy of the intestine where ISEMF situated below the epithelium monolayer. The difference in the paracrine signal gradient received by the enteroids due to its curvature might adds some complexity to our understanding the interaction between the epithelium and the underlying ISEMF [14]. Few studies have investigated effect of ISEMF on the development of the intestinal epithelium utilizing intestinal monolayer culture platform. Puzan et. al. recently showed that through culturing a feeder layer of mice ISEMF at the bottom of the well, the mice intestinal monolayer in the Transwell insert exhibit better proliferation and monolayer integrity with increased transepithelial electrical resistance (TEER) value. Additionally, certain level of cellular differentiation was also observed with more enteroendocrine cells presented in the co-cultured model. A similar study done by Altay et. al. [16] also demonstrated similar results where when co-cultured with mouse ISEMF, the mouse intestinal epithelial monolayer was able

to reached full confluency faster with increase berried function. These studies provided a preliminary insight on the effect of ISEMF on the development of the intestinal epithelial monolayer, however, these studied were all done with cells form mice. It is believed to be imperative to further investigate whether the same effect of the ISEMF was conserved on the development of the human intestinal epithelial monolayer as well as its underlying mechanisms.

In this study, we sought to investigate the effect of the ISEMF on the development of human intestinal epithelial monolayer and shed some light on its underlying mechanisms by using Transwell cultured human intestinal epithelial monolayer.

4.2 Materials and Methods

Isolation of human intestinal crypts

Small intestinal samples were obtained fresh from the Surgical Pathology Department. Tissue was removed from PBS solution and washed multiple times with ice-cold PBS until the solution remained clear. The specimen is placed in a Petri dish containing PBS on ice with the mucosal surface facing upward. Mucosectomy is then performed with surgical scissors and forceps. The mucosa is divided into approximately 1 x 1 cm pieces and washed with cold PBS via vortexing for 30 seconds with three 10-second pulses until the supernatant is clear. These pieces were then incubated in 8 mM EDTA and 1mM DTT solution in PBS for 30 minutes with gentle shaking at 4 ˚C. After the incubation period, the fragments were allowed to settle and the supernatant was discarded. 30 ml of cold PBS is added to the sample, and subsequently vortex for 30 seconds with 3-second pulses. 15 ml of the supernatant was removed and save on ice. Again 15 ml of PBS were added and the process was repeated six times. Six fractions were spun down at 100 g for 2 minutes. The supernatant was discarded. The contents of the pellets were examined under light microscopy. Typically, all fractions were pooled together to increase yield of epithelial crypts.

The pooled fractions were then purified using a 100 μm and 70 μm pore filters. The pooled fraction after filtration would be crypts-rich suspended solution.

Maintenance of human intestinal stem cells

Every 250 crypts are suspended in 25 μL Matrigel as described in Sato's 3-D Matrigel culture system developed for murine intestines [17] The 25 μL of crypt cell/Matrigel suspension is placed in 48-well plate. Matrigel (Fisher) was allowed to polymerize in the incubator for 15 minutes. 250 μL/well of proliferation medium will then be added to the wells. 10 μM Y-27632 dihydrochloride (Fisher) and 2.5 μM CHIR-99021 (Fisher) were added into the proliferation media for the first two days after passage. The enteroids were maintained in 37°C humidified incubator with 5% carbon dioxide until ideal number of enteroids was reached. The media was changed every two days. At the time of passaged, the enteroids containing Matrigel buttons were dislodged with P1000 pipettes and along with media, collected into 1.5 mL Eppendorf tubes. The pellets were acquired via microcentrifuge with three 3-second pulses. The Matrigel containing pellets were then digested with 500 μL of TrypLE Select disassociation reagent (Thermo Fisher) in 37°C waterbath for 5 minutes. The disassociation reagent was then quenched with DMEM with 10% FBS and 1X AMAB (Life Technologies). After quenching, the enteroids were further broken down mechanically via pipetting the content with P1000 pipettes repeatedly. The pellets were then acquired via microcentrifuge with three 3-second pulses. The pellets were then resuspended with desirable amount of Matrigel and re-seeded into a new tissue plate

ISEMF culture and conditioned media generation

Intestinal subepithelial myofibroblast (ISEMF) culturing and conditioned media generation protocol was described in previous publications [11]. In short, ISEMF was cultured in tissue culture flask with Dulbecco's Modified Eagle's Media (DMEM, Life Technologies), with 10% FBS and 1X AMAB (Life Technologies), supplemented with 20 ng/mL EGF (Peprotech), 10 μg/mL

Transferrin (Sigma) and 5 µg/mL Insulin (Sigma). The media was changed every other day. The ISEMF was cultured in humidified tissue culture incubate at 37°C, 10% carbon dioxide. Once reached full confluency, the ISEMF was passaged with TrypLE select (Fisher) in seeding density of 500k/flask (T75).

To generate ISEMF conditioned media, the ISEMF was seeded at 500k/flask (T75) seeding density and cultured for 7 days without media change. After one week, the conditioned media were collected, filtered with 0.2 µm filter and stored in 4°C.

Culture with ISEMF condition media

The method of culturing enteroids with ISEMF conditioned media was described previously in the publications [18] In short, the monolayer was cultured with PM-ISEMF_CM which consisted of 50/50 mix of Advanced DMEM/F12 (Invitrogen) and ISEMF conditioned media (ISEMF_CM). The mixture is also supplemented with 1mM N-Acetylcysteine (Sigma), 2mM GlutaMax-1 (Invitrogen), 10mM HEPES buffer (Invitrogen), 1X ABAM (Initrogen), 1X B27 (Invitrogen), 1X N2 (Invitrogen), 50 ng/mL EGF (Peprotech), 100 ng/mL Noggin (Peprotech), 1 µg/mL R-spondin1 and 10 µM Y-27632 dihydrochloride (Fisher). The media was changed every other day.

Generation of intestinal monolayer and ALI

Transwell culturing method was adopted from Julie In et al [19]. The 24-well Transwell inserts (Corning 3414) with polycarbonate membrane were coated with collagen IV (30 µg/mL) prior cell seeding.

The enteroids-containing Matrigel buttons were scraped off from the tissue culture plate with P1000 pipettes and washed with 0.5 mM EDTA/PBS (Sigma). After pelleting the content via centrifuging at 1000 rpm for 5 minutes, the Matrigel/enteroids were then disassociated chemically with 0.25%/0.5mM Trypsin/EDTA in 37 ˚C water bath for 5 minutes. After quenching

with DMEM/10% FBS/1X ABAM, P1000 and P200 pipettes were used to break apart the enteroids mechanically, ensuring the enteroids were disassociated into single cells. Cell containing solution were then pass through 40µm cell strainer before centrifuging at 1000 rpm for 5 minutes.

cell pellets were resuspended with proliferation media supplemented with Y-27632 dihydrochloride and CHIR-99021. The cells were seeded into the collagen IV coated Transwell inserts at a seeding density of 750k/cm^2.

The monolayers would be cultured in proliferation media with 100 µL in the insert and 600 µLin the well with media change every 2 days.

After the monolayer has been cultured with proliferation media 5 days ensuring the monolayer has reached full confluency, Air-Liquid Interface (ALI) culturing condition would be initiated. For the first 5 day of the experiment, the monolayer was cultured with 100 µL of media covered it, the culturing condition was denoted as "Normal culturing condition". On day 5, to start ALI culturing condition, the media on top of the monolayer would be removed, leaving the monolayer in direct contact to the environment, while the media in the well would be reduced to 350 µL. The media was changed every other day.

Co-culture system

For the co-cultured system, Both sides of the Transwell membrane were coated with collagen IV (30 µg/mL) prior cell seeding.

ISEMFs were disassociated from the tissue culture flasks and collected. The cells were resuspended in ISEMF culture media and seeding on the outside of the Transwell membrane at 303k/cm^2 seeding density. The seeding was done by inverting the Transwell insert so the outside of the membrane facing upward. The cell suspended droplets (100 µL) were then dropped on top

of the membrane and incubated in humidified tissue culture incubate at 37°C, 10% carbon dioxide for at least 2 hours to ensure proper cell attachment. After 2 hours, the inserts were then inverted back and placed back into the 24-well tissue culture plate and supplemented with ISEMF culture media in both inserts and the wells. The plate was them place back into the incubator for overnight.

After 24 hours, followed the monolayer generation process described above to seed the enteroids to generate monolayer and subsequent ALI culturing condition. The culture media was then switched to proliferation media.

BMP4 treatment

The monolayer was cultured with proliferation media for five days after being seeded into the Transwell inserts. On day 5, 30 ng/mL of recombinant BMP4 was added into the proliferation media and ALI culturing condition was initiated at the same time. The media was changed every other day.

Immunohistochemistry

A.   *Sample preparation for histology*

The monolayers were fixed by adding 200 µL of 10% buffered formalin (Fisher) into the inserts for 30 minutes in room temperature. The monolayers were washed with PBS twice after the fixation. The samples needed to be embedded within Histogel (Fisher) in an upright fashion. Liquified the Histogel by warming it up to 65°C and was kept warm for it to remain at liquid state. The polycarbonate membrane was carefully cut off with scalpels. The membrane was them cut into four strips with razor blades and stacked one on top of each other. Place a lid of the 10 cm diameter petridish on an ice block and, with transfer pipette, drops couple droplets of liquified Histogel onto the lid for form a button. Once the Histogel button was solidified due to the drop of the temperature, cut a slit on the button and place the membrane strip into the slit of the Histogel

in an upright position so when sectioning, the cross section of the monolayer could be observed. The Histogel button was then covered with fresh Histogel ensuring the strip was covered with Histogel. The button was then leaved on the ice block for 15 minutes before transferring into -20 °C for 10 minutes. Lastly, the strip containing Histogel button was then transferring to the histology cassette and stored in 70% ethanol before submitting to the Stanford Human Pathology/Histology Service Center for paraffin embedding, sectioning and Hematoxylin and Eosin staining (H&E).

B.   *Immunostaining of differentiation marker*

Unstained slides were sectioned at 4 μm thickness with 2 sections per slide. Slides were washed with xylene for 5 minutes twice to remove the paraffin wax. The sections were then rehydrated with progressive decreasing concentration of ethanol in water from 100% to 70% for 2 minutes and finished with 5 minutes incubation in deionized water. Antigen retrieval was done by incubate the slides in 1X Antigen Retrieval Citra Solution (Fisher) for 15 minutes at 100 °C followed by 20 minutes cooling in cold water bath. Samples were then permeabilized with 0.5% Triton-X for 5 minutes and washed with PBS-Tween twice afterwards. A hydrophobic barrier was created with PAP pen (Abcam) before covering the sections with blocking solution containing 2% bovine serum albumin and 5% Normal goat serum for 1 hour in room temperature to prevent non-specific staining. Following blocking, primary antibody working solutions with primary antibody diluted in blocking solution at a ration which is antibody specific was added on top of the designated sections. The sections were incubated at 4 °C overnight. Primary antibodies used would be Muc2 (Santa Curz), E-cadherin (Santa Cruz, abcam), ki67 (Santa Cruz), villin (Santa Cruz). Next day, the slides were washed with PBS-Tween three times to wash away excess primary antibody before adding secondary antibodies which was diluted in PBS-Tween at 1:50 ratio on top of the sections. For the secondary antibodies, Alexafluor 488 goat anti-mouse, Alexafluor 488 goat anti-rabbit, Alexafluor 594 goat anti-mouse or Alexafluor 594 goat anti-rabbit were used depends on

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the primary antibodies used at 1:200 ratio. Excessive secondary antibodies were washed away with three PBS-Tween wash before adding mounting media with DAPI (Fluoroshield with DAPI, Sigma) was added. Fluorescent images were taken with Olympus microscope with CellSens software (Olymous).

C. *TUNEL assay*

The In situ BrdU-Red DNA Fragmentation (TUNEL) assay was performed following the protocol provided by the vendor (ab66110, Abcam) with the exception of 0.5% Triton-X instead of protease K treatment. The samples were counterstained with DAPI at the end

D. *Quantification of ki67 and TUNEL*

Immunofluorescent pictures of each samples with ki67 staining and TUNEL assay treatment were taken. Three randomly chosen areas of each slides were taken. Positive ki67 cells were only counted when ki67 and DAPI signals were colocalized. TUNEL positive cells were only counted when its signal was colocalized with DAPI. (n=3 for 3 independent experiments)

Analysis of RNA expression

RNA extraction was described in a previous publication [20]. Primer and probe combinations were purchased from Applied Biosystem (Taqman Expression Asssay, Hs00969422_m1(LGR5), Hs00894052_g1(muc2), Hs00167206_m1(SLC11A2), Hs00426232_m1(lyz), Hs00900375_m1(Chga), Hs01031724_m1(vil1), Hs02758991_g1(GAPDH), Hs99999142_m1(CDKN1A). RT-PCR was performed in accordance with the description in a previous publication [20] with ABI 7900HT Fast Real Time PCR system. Cycle numbers of all samples were normalized to GAPDH with human intestinal (whole bowel (WB) serving as control tissue.

Statistics

Differences between groups were evaluated via Student's t-test. A p-value < 0.05 was considered as statistically significant.

4.3 Result

Immunohistochemistry of co-cultured monolayer

From the H&E staining, the human ISEMF can be seen on the opposite side of the membrane (Fig. 4-1). The morphology of the epithelial monolayer is more organized and was able to maintain its integrity throughout the experiment. Furthermore, the effect of ALI on the morphology of the monolayer can still be seen where the cells became columnar.

The images showed that the cells at the other side of the membrane were ISEMF with positive SMA staining while the cells on the opposite sides were epithelial cells with negative SMA and positive E-cadherin staining (Fig. 4-2).

Ki67 / TUNEL analysis

Ki67 positive cells were present throughout the experiment (Fig. 4-3). This would be a great advantage over monolayer cultured with differentiation media where all the proliferative cells were transformed into secretory cell lineage.

Additionally, upon examining the cellular apoptosis, the cells seem to experience less apoptosis overall (Fig. 4-4). Furthermore, the cells cultured under ALI condition exhibit even less cellular apoptosis than its counterparts cultured under submerged condition.

After close examination of the percentages of ki67 positive proliferative cells and TUNEL positive apoptotic cells quantitatively, it revealed that even though no significant differences in proliferative
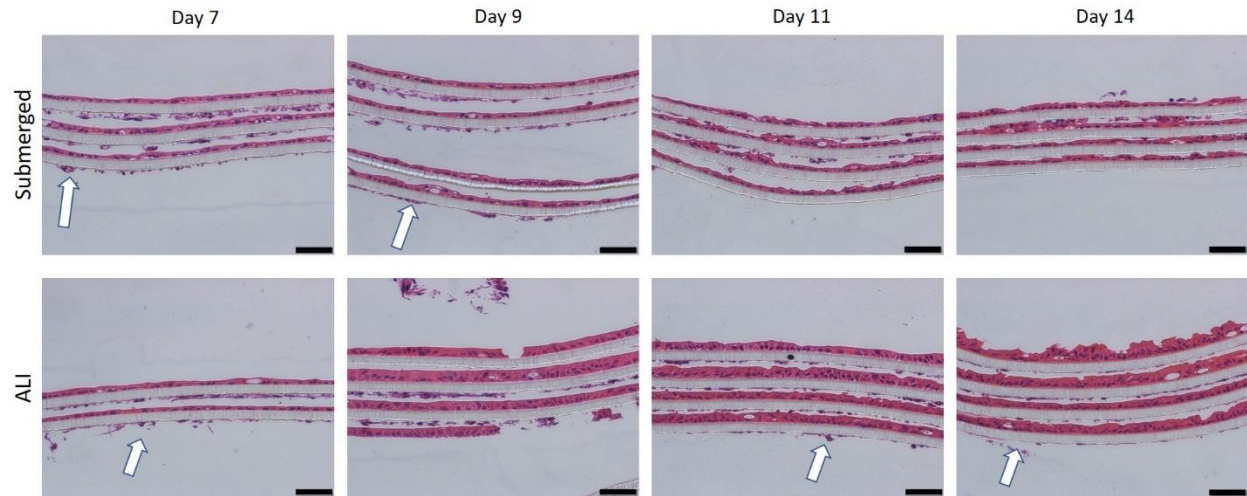
Figure 4-1: H&E histology of the epithelium co-cultured with ISEMF on the other side of the Transwell membrane (arrow) under two culturing conditions. The epithelium was more organized than monoculture. ALI had the same effect on cellular morphology where the epithelial cells adopted columnar shape when co-cultured. Scale bar: 50 µm
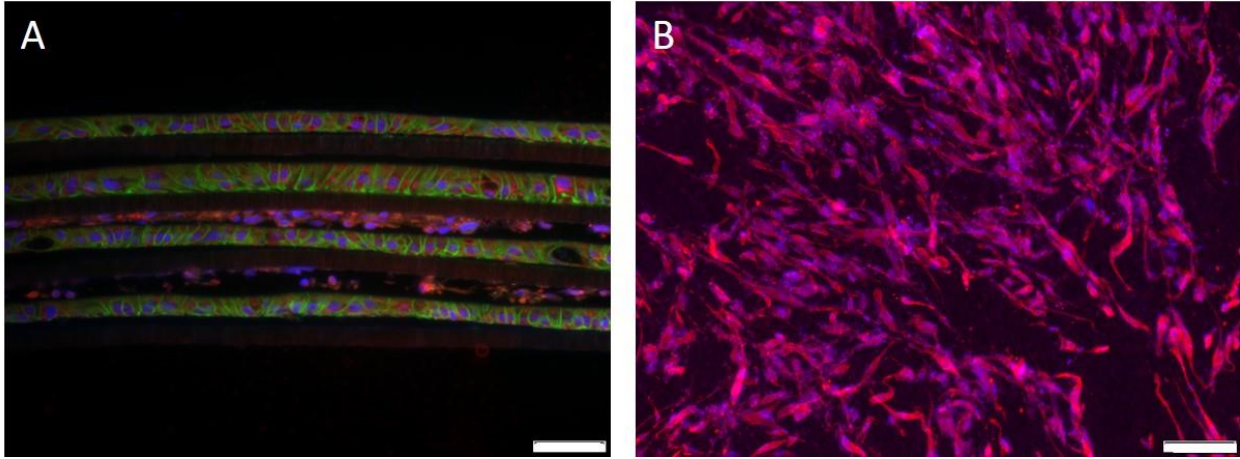
Figure 4-2: (A) Cross sectional view of immunofluorescence of SMA expression on the co-cultured model. SMA positive (red) ISEMF could be seen on the opposite of the e-cadherin (green) positive SMA negative epithelial layer. Scale bar: 50 µm. (B) Top-down view of SMA positive (red) ISEMF on the Transwell insert membrane. Scale bar: 50 µm
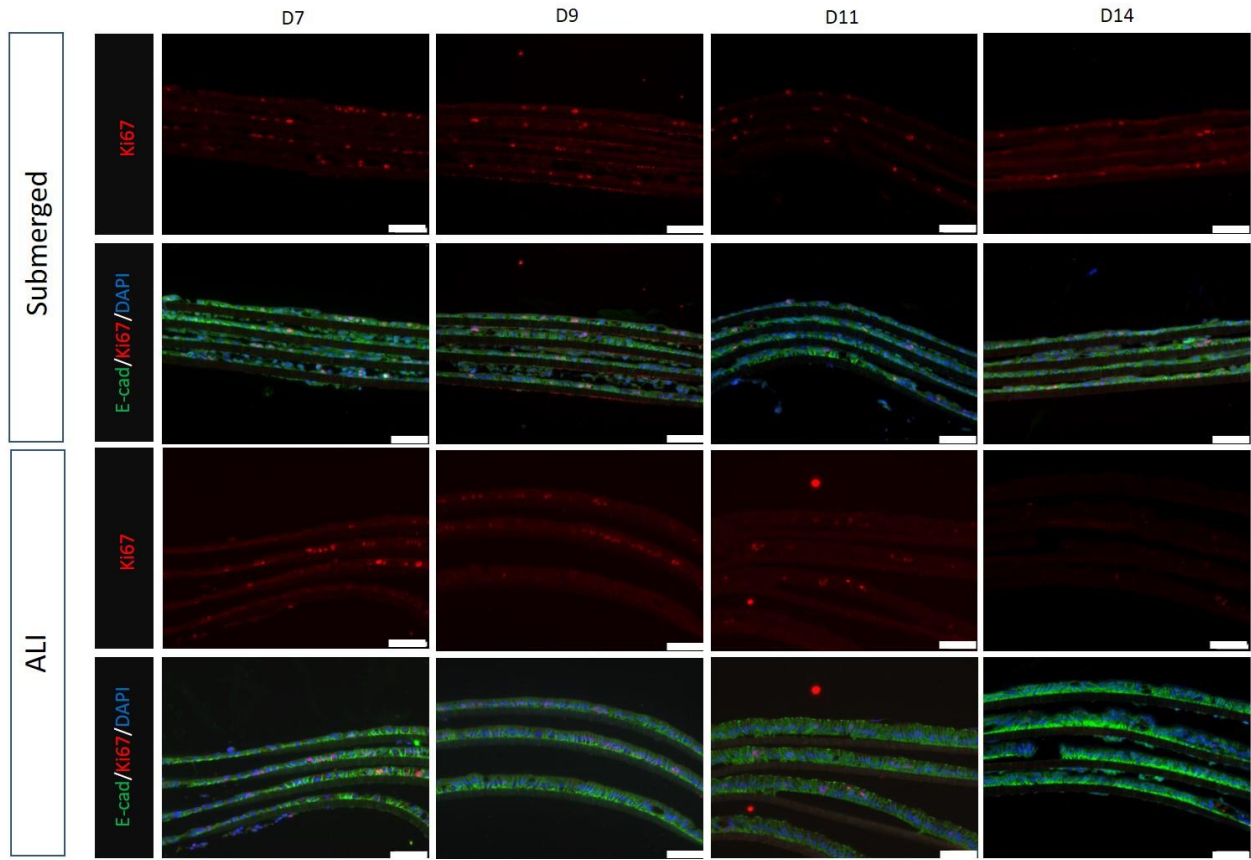
Figure 4-3: Immunofluorescence of indirect co-cultured epithelial monolayer from day 7 to day 14 under two culture conditions. Ki67 positive cells could be seen in both culturing conditions. Scale bar: 50 µm
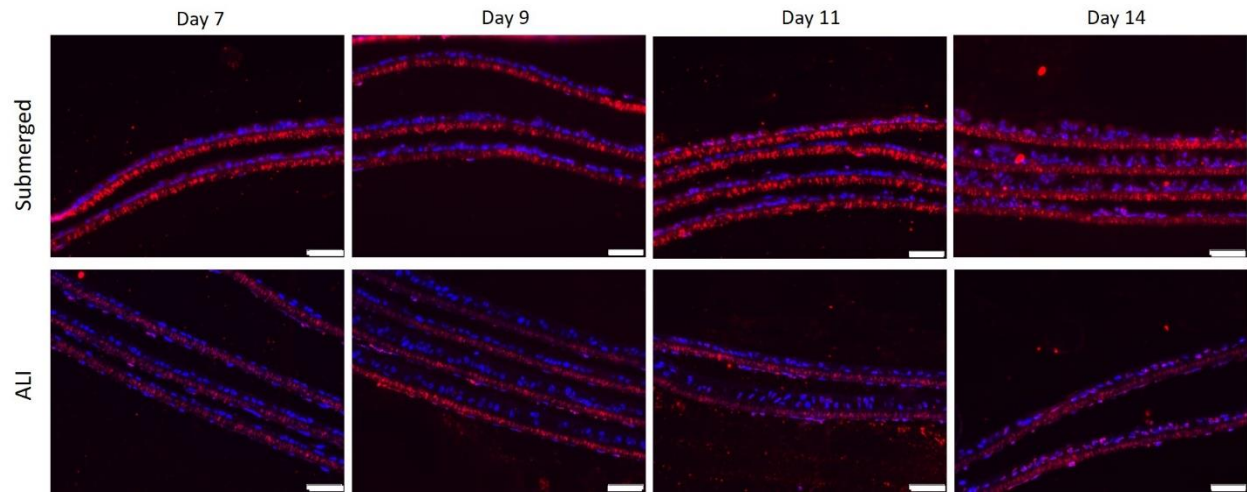
Figure 4-4: TUNEL assay on the co-cultured monolayers culturing under two culturing conditions. Scale bar: 50 µm

cells between Normal and ALI condition throughout the experiment, there is significant differences in the percentages of apoptotic cells (Fig. 4-5).

The percentages of apoptotic cells when cultured under ALI condition were significantly lower than the ones in submerged condition at early time point (Day 7, Day9). The possible reason to the lack of difference at the later timepoints was due that the ISEMF have started to detach from the membrane since they were not cultured with ISEMF culture media after co-culture was initiated. Therefore, the effect of the ISEMF on the epithelial monolayer might started to decrease as the ISEMF started to detach.

When co-culture with ISEMF, the percentage of apoptotic cells in submerged condition was comparable to the one in human intestine (Fig. 4-6). This is likely part of the reasons to the difference in monolayer integrity between mono-culture and co-culture.

After comparing TUNEL assay results between mono-culture and co-culture, it exhibited a dramatic decrease in the numbers of apoptotic cells when monolayer was cultured with ISEMF (Fig. 4-7).

Through quantitative analysis of cellular apoptosis between mono-culture and co-culture, the effect of ISEMF on cellular apoptosis could clearly be seen (Fig. 4-8). Through co-culture with ISEMF, cellular apoptosis decreased in both submerged and ALI culturing conditions. This result further supported the hypothesis that the decrease in cellular apoptosis might be one of the reasons to the more organized monolayer when co-cultured with ISEMF.

Comparison between different co-culture method

To investigate whether the distance between ISEMF and epithelial monolayer plays a role in the efficacy of the co-culture, ISEMFs were seeding in the wells instead of the opposite side of the membrane. In this case, there was 3 mm distance between the ISEMF and the epithelial
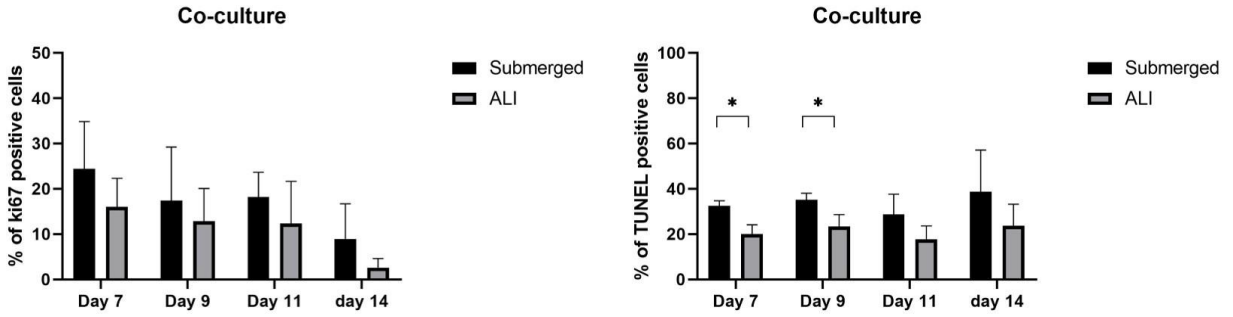
Figure 4-5: Quantitative comparison in Ki67 positive cells and TUNEL positive cells of the co-cultured monolayer. No significant difference was observed. ALI cultured monolayer showed significant decrease in apoptotic cells. (* $p < 0.05$). (n=3)
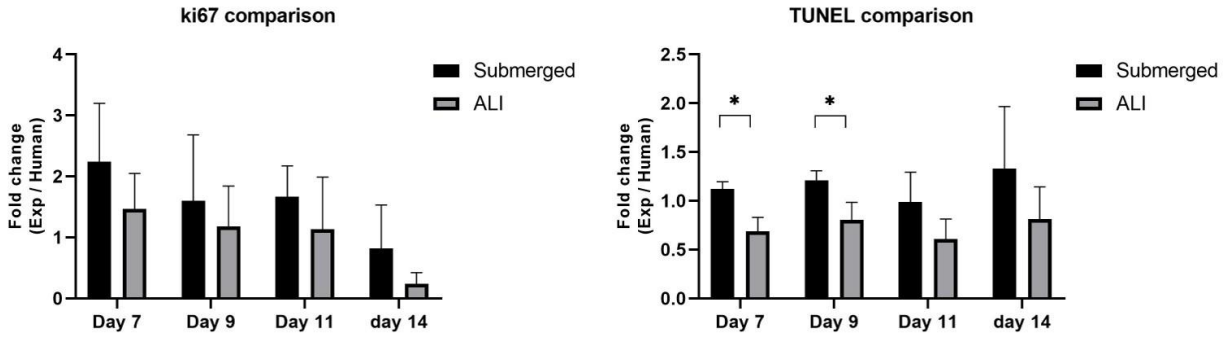
Figure 4-6: Quantitative comparison on ki67/TUNEL between the in vitro co-cultured monolayer and human intestinal epithelium (n=3)
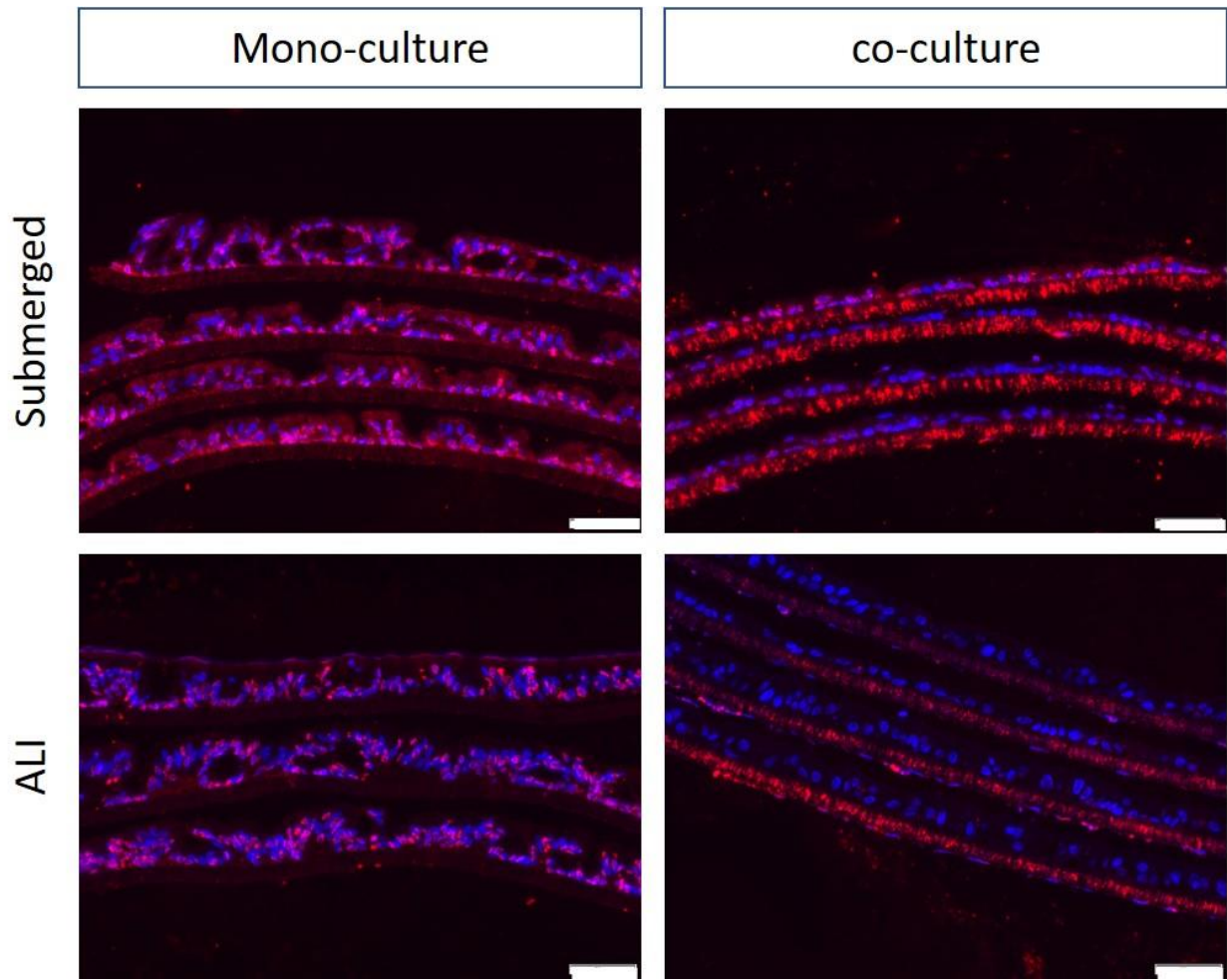
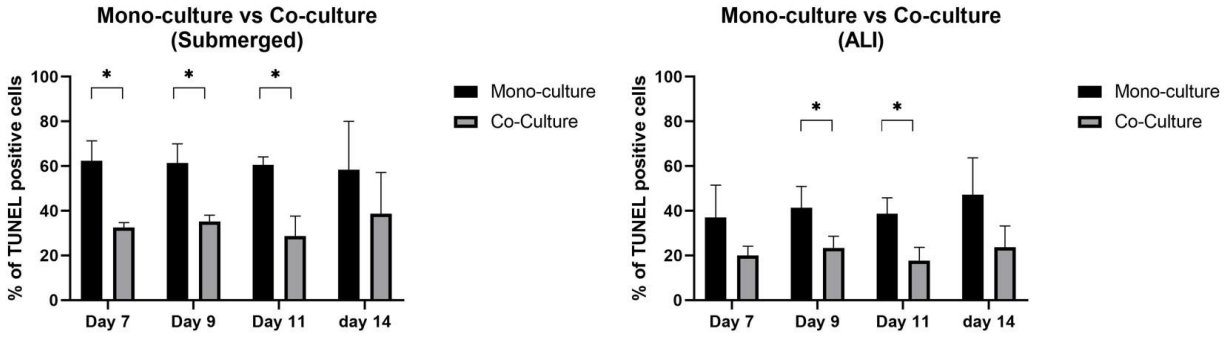Figure 4-7: TUNEL assay comparison between mono-culture and co-culture.   Scale bar: 50 µm

Figure 4-8: Quantitative comparison of TUNEL positive cells between mono-cultured and co-cultured monolayer. There was significant difference between the two under both Submerged and ALI culturing condition. (* $p < 0.05$) (n=3)

monolayer which situated inside the Transwell inserts. The results showed that the monolayer also took on a more organized morphology throughout the experiment, especially the one under ALI. The columnar shaped morphology was observed (Fig. 4-9).

After examining the differentiation of the monolayer under in-direct co-culture condition, the increased number of Muc2 positive cells were observed compared the one cultured with proliferation media (Fig. 4-10), suggesting that the monolayer was more differentiated. Additionally, more Muc2 positive cells were present under ALI condition than submerged condition support previous conclusion that ALI has an effect of heighten the differentiation of the monolayer, provided there is an existing differentiation cue. In this case, the cue may be coming from ISEMF since more Muc2 positive cells were also observed in the monolayer culture under submerged condition.

On the other hand, the distance between ISEMF and monolayer seems to have an effect on the cellular differentiation. From the immunostaining results, the number of Muc2 positive cells was significantly less compared to in-direct co-culture (Fig. 4-11).

Immunostaining of the monolayer

At the same time, the formation of brush border was also observed on the monolayer that had been cultured under ALI condition with Villin localized at the apical region of the monolayer under ALI culturing condition (Fig. 4-12). The result suggested that ALI has the same effect across multiple different culture conditions.

RNA expression analysis

RNA expression analysis via RT-PCR showed that other than lysozyme, villin and SLC11a2 exhibit decent expression, there was significant increase in the Muc2 expression when ALI cultured which correlate with the immunostaining results (Fig. 4-13). Again, the phenomenon
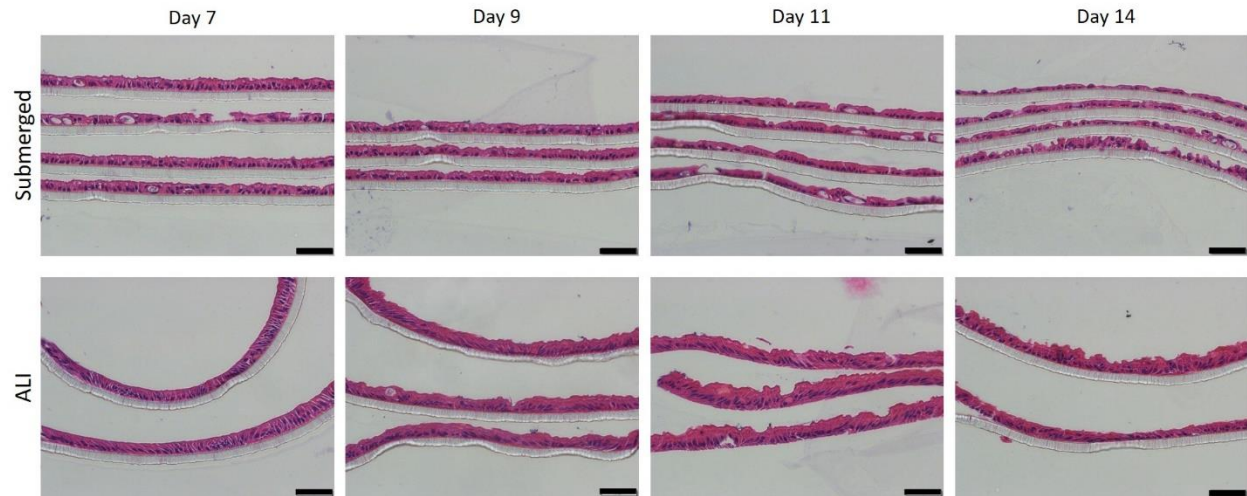
Figure 4-9: H&E histology of the epithelium co-cultured with ISEMF at the bottom of the well under two culturing conditions. Similar phenomenon on cellular morphology with co-cultured with ISEMF on the other side of the membrane. ALI also had similar effect on cellular morphology. Scale bar: 50 µm

Figure 4-10: Immunofluorescence of the epithelial monolayer against Muc2 (red) and e-cadherin (green) of co-cultured with ISEMF on the opposite side of the Transwell insert membrane from day 7 to day 14 under two culture conditions. Muc2 positive goblet cells could be seen on the epithelial monolayer on both conditions. There were more goblet cells presented on the monolayer cultured under ALI condition.

Scale bar: 50 µm
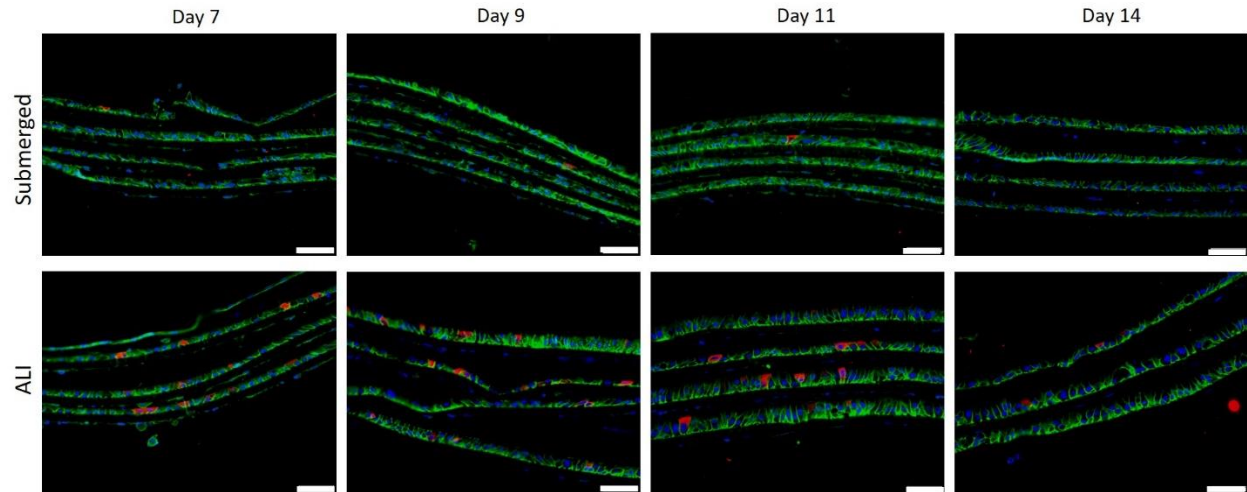
Figure 4-11: Immunofluorescence of the epithelial monolayer against Muc2 (red) and e-cadherin (green) of co-cultured with ISEMF in the well from day 7 to day 14 under two culture conditions. Muc2 positive goblet cells could be seen on the epithelial monolayer on both conditions. There were more goblet cells presented on the monolayer cultured under ALI condition. Scale bar: 50 µm

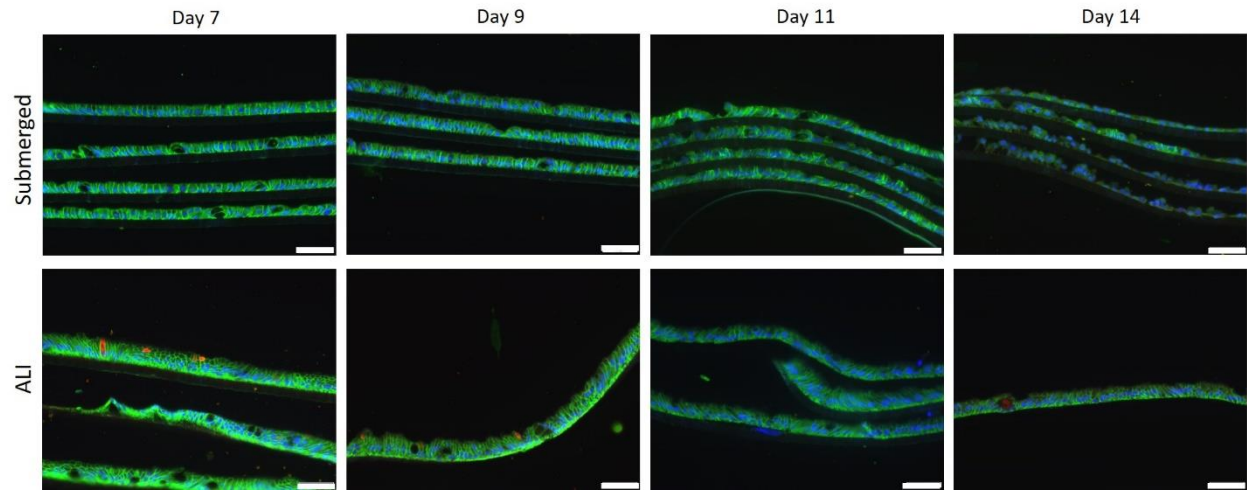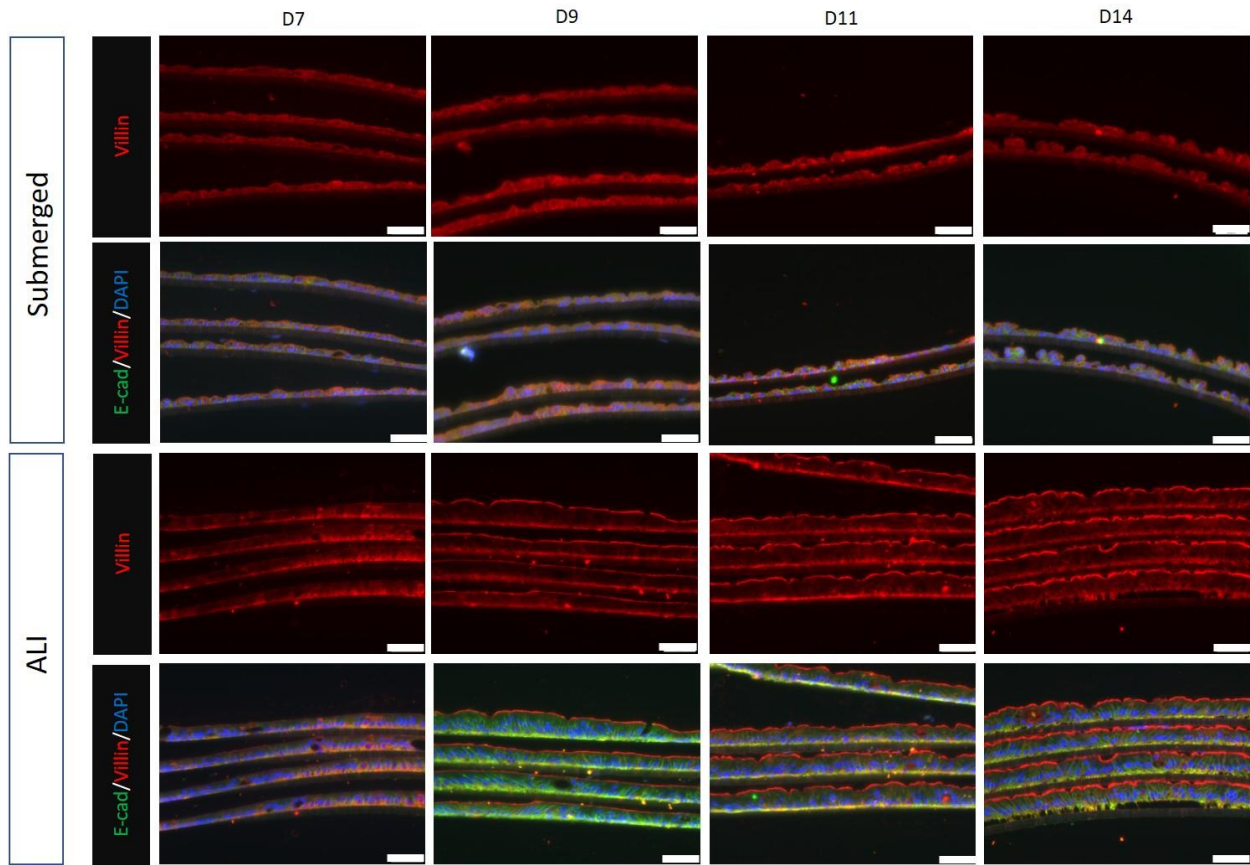Figure 4-12: Immunofluorescence of co-cultured epithelial monolayer from day 7 to day 14 under two culture condition. Villin signal can been seen localized at the apical side of the monolayer under ALI condition. Scale bar: 50 μm
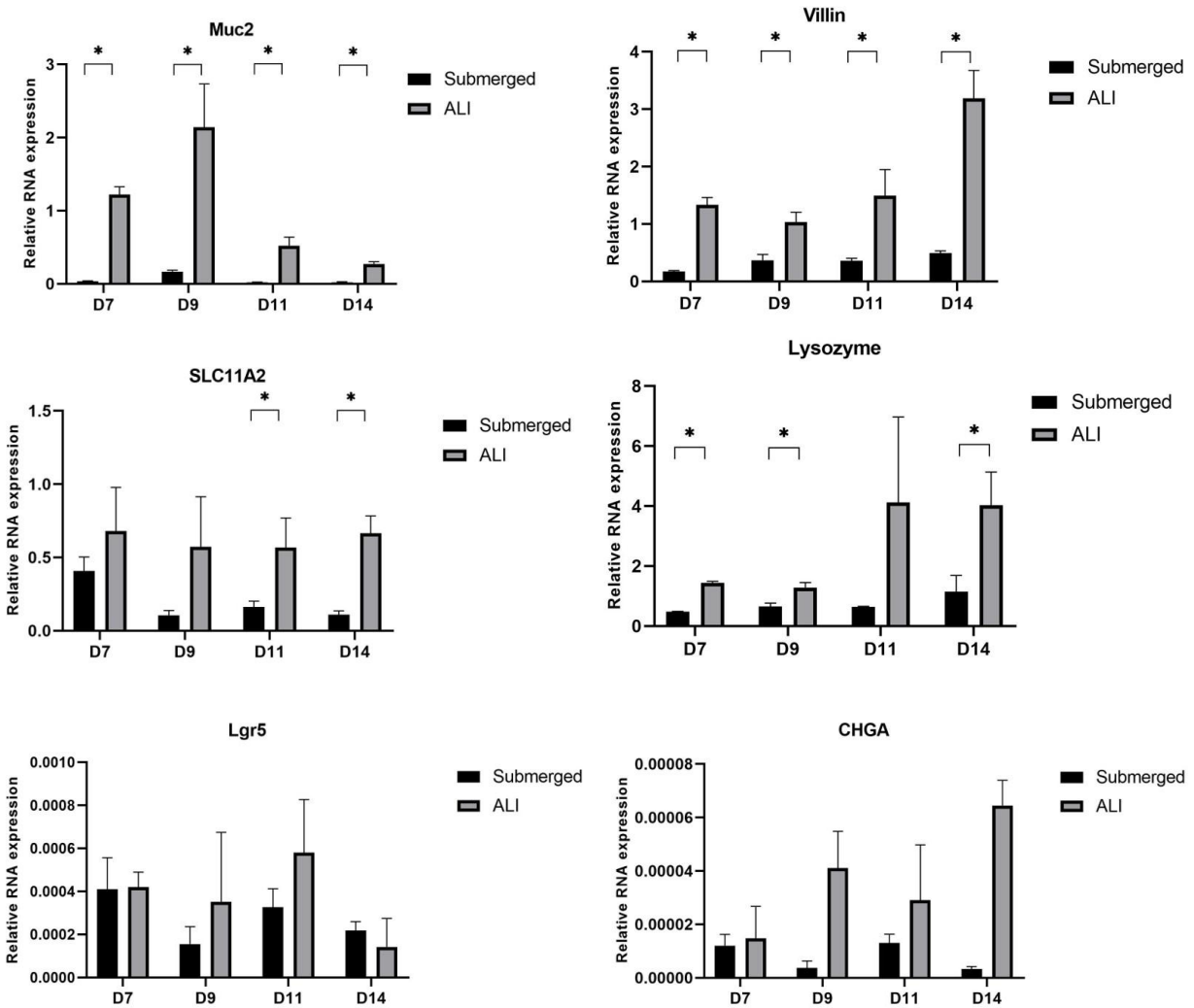
Figure 4-13: RT-qPCR analysis on multiple differentiation marker for the epithelial monolayers. Muc2 expression significantly increased compare to mono-culture. The expression of Muc2, Lysozyme, Villin and SLC11a2 showed significantly higher expression when culturing under ALI condition. CHGA and Lgr5 also showed the same trend, however, overall expression remained low. Human intestine was used as control. (n=3). (* p < 0.05)

where the expression of ALI cultured monolayer consistently exhibited higher expression than submerged cultured monolayer still persisted in co-cultured scenario.

Monolayer development with ISEMF conditioned media

One way to simplify the co-culture system was the use of ISEMF conditioned media. It has been reported that 1:1 mixture of ISEMF conditioned and basal culture media, here denoted as PM_ISEMFCM, was able to support the growth of the enteroid as well as monolayer on the tissue culture plates. This concept was employed here to culture intestinal monolayer on the Transwell insert long term. From the H&E images showed here indicated that the ISEMF conditioned media has the same effect on the development of the monolayers (Fig. 4-14). The morphology of the monolayer was extremely organized and the cells under ALI condition adopted columnar shape as before.

ISEMF conditioned media also has the same effect in cellular differentiation as co-culture. ISEMF conditioned media was able to provide differentiation cue to the monolayer hence the presence of the Muc2 positive cells. At the same time, ALI culturing condition was able to amplify the differentiation cue resulted in much more Muc2 positive cells than its counterpart in submerged culturing condition (Fig. 4-15).

Investigation of Bone Morphogenetic Protein (BMP) signaling activity on monolayer development

Several studies have shown that as part of the intestinal stem cell niche, ISEMF affects the stemness and epithelial cell differentiation through Bone Morphogenetic protein (BMP) pathway. There exists a concentration gradient of BMP within crypt-villus axis. To further explore if BMP played a role in this co-culture system where there was more differentiation and less cellular apoptosis, the ISEMF condition media culturing system was used. With this culture system, the amount of Noggin, an antagonist to BMP pathway, can be manipulated and subsequently affecting the activation of the BMP pathway.
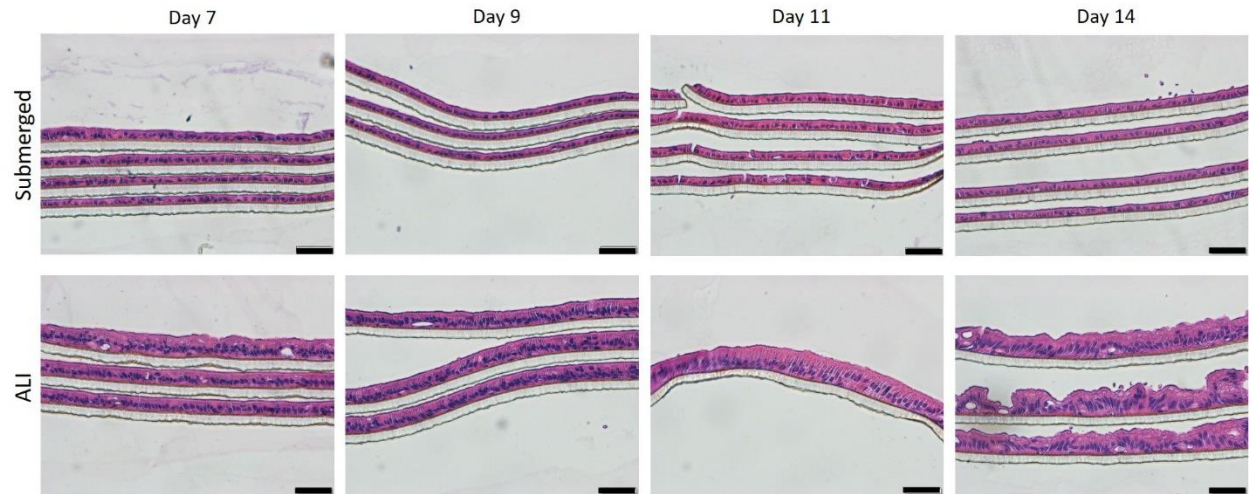
Figure 4-14: H&E histology of the epithelium cultured with PM_ISEMF CM. The result showed ISEMF CM had similar effect on monolayer development as co-cultured with ISEMF. Scale bar: 50 μm
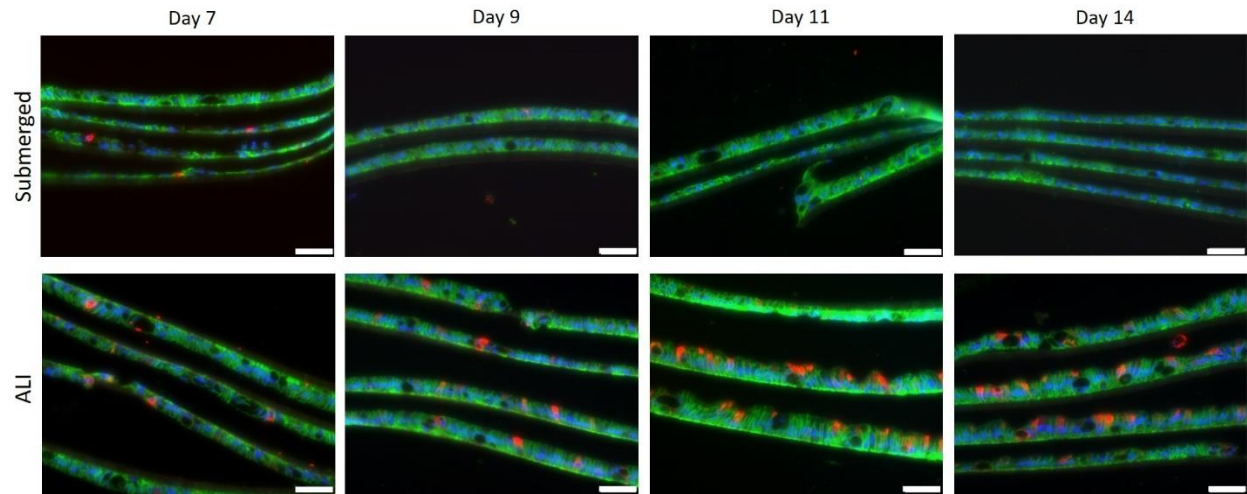
Figure 4-15: Immunofluorescence against Muc2 (red) on monolayer cultured with PM_ISEMF

CM. Muc2 positive goblet cells can be seen. Scale bar: 50 µm

Different concentration of Noggin was used to inhibit the BMP pathway activity at different level. From the H&E results, there seems to be little to no difference in terms of the morphology of the monolayer when cultured under submerged condition (Fig. 4-16). However, when culturing under ALI condition, the effect of varying the concentration of Noggin on the monolayer development could be observed (Fig. 4-17). The monolayer was less organized without any Noggin in the culture medium comparing to the one with 400 ng/mL of Noggin.

Upon examining the cellular differentiation with Muc2 antibody, the results showed that the number of MUC2 positive cells decreased in both normal and ALI condition as the concentration of the Noggin increased (Fig. 4-18).

On the other hand, the number of ki67 positive proliferative cells increased as the concentration of the Noggin increased (Fig. 4-19).

The decrease in the numbers of Muc2 positive cells and increase of ki67 positive cells as the concentration of Noggin increased can be seen clearly from the quantitative results (Fig. 4-20). In every time points and under both culturing condition, the increase of ki67 positive cells and decrease of Muc2 positive cells as the concentration of Noggin increased remained a consistent phenomenon. These results suggested that BMP pathway indeed played a role when epithelial cells and ISEMF were co-cultured. The differentiation cue likely came from BMP pathway induced by ISEMF.

Many factors involved in the activation of BMP pathway. There are BMP4, BMP2, Smad1/3/5 compound…etc. In efforts to identify the exact mesenchymal factor that was involved in activation of the BMP signaling pathway in co-culture system, the role of BMP4 was examined. The recombinant BMP4 was added into the monolayer culture system with proliferation media. The morphology and the differentiation state of the monolayer was examined.
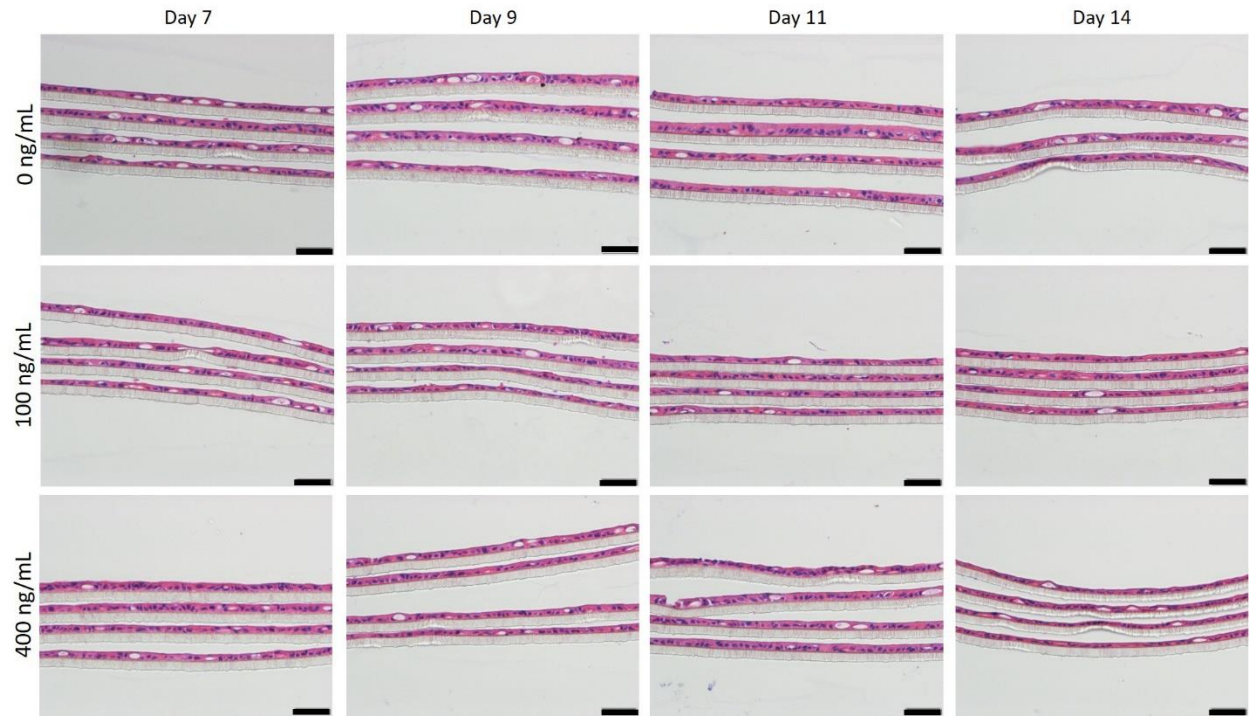
Figure 4-16: H&E histology of the epithelium cultured with various concentration of recombinant Noggin under submerged culturing condition. Scale bar: 50 µm
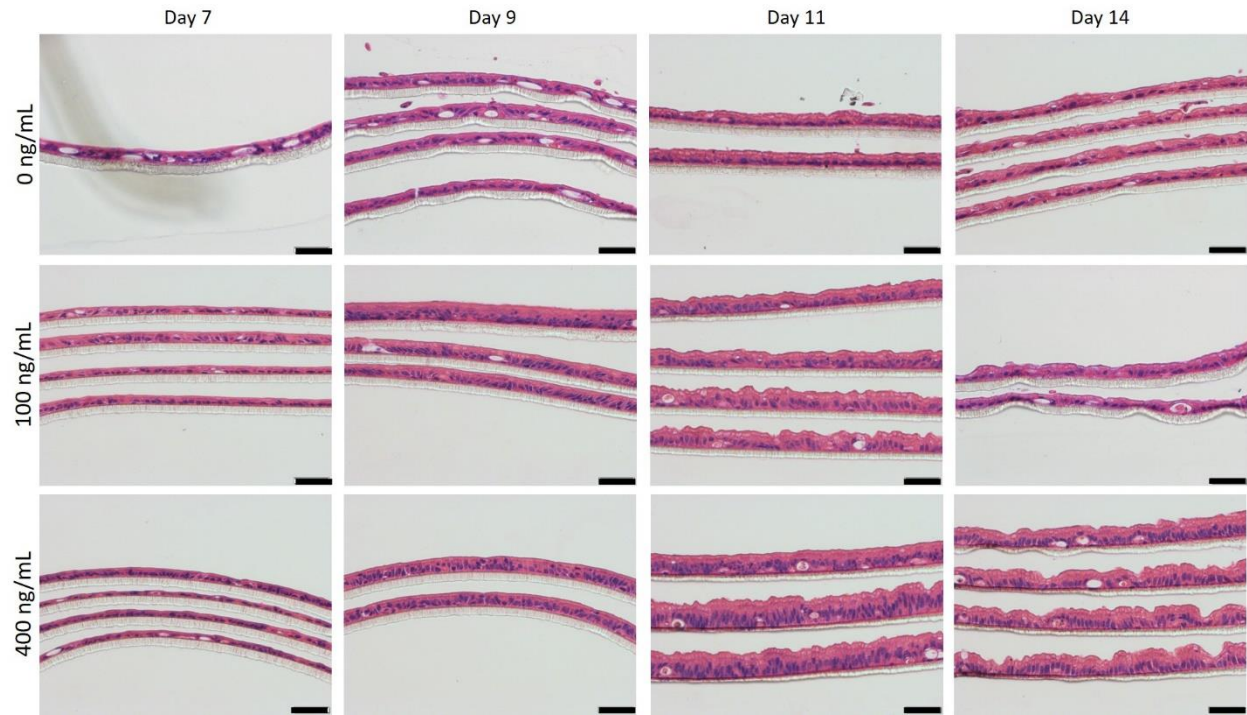
Figure 4-17: H&E histology of the epithelium cultured with various concentration of recombinant

Noggin under ALI culturing condition. Scale bar: 50 µm
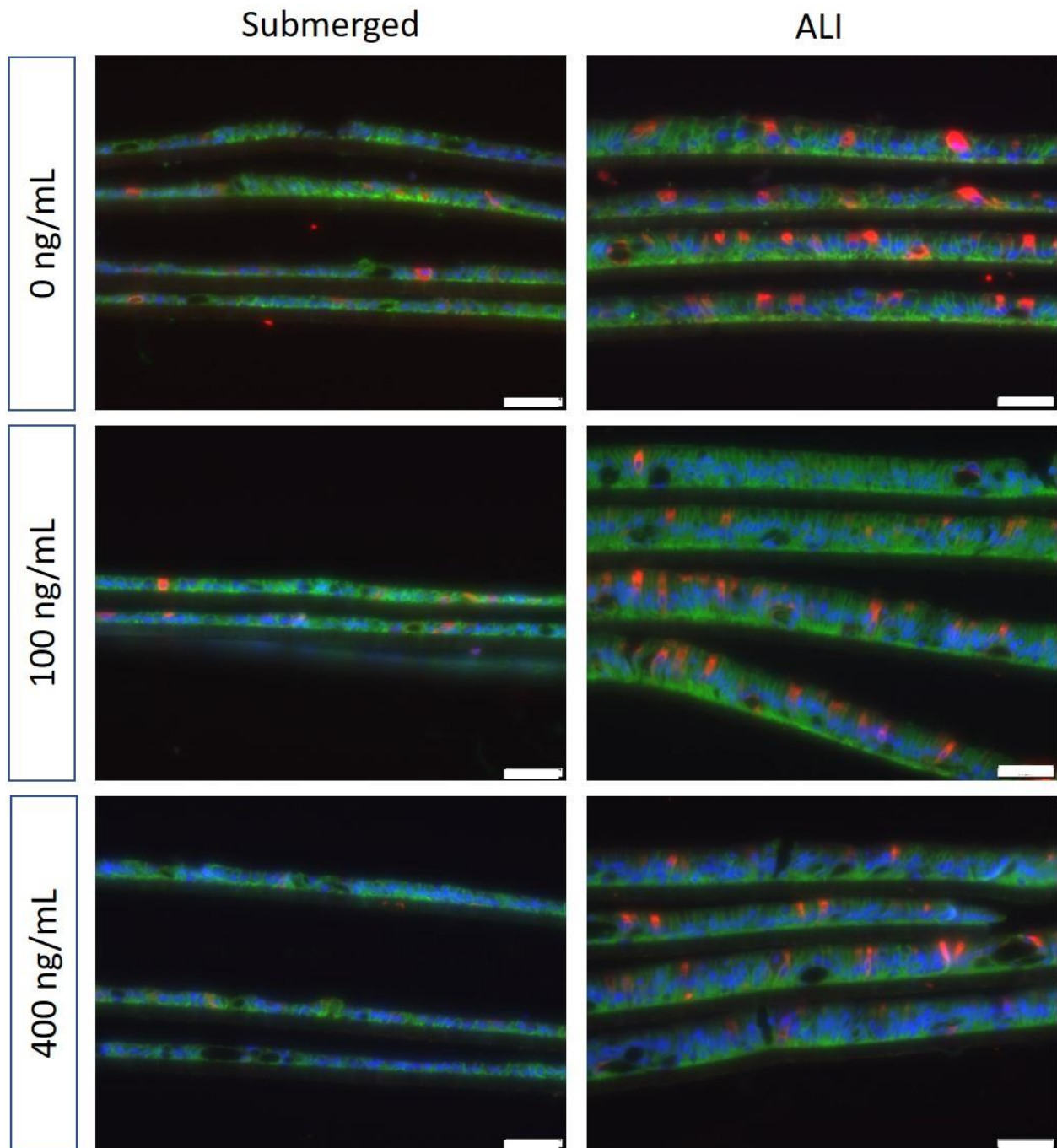
Figure 4-18: Immunofluorescence against Muc2 (red) on monolayer cultured with various concentration of Noggin. It showed that as the concentration of Noggin increased, Muc2+ cells decreased. Scale bar: 50 μm
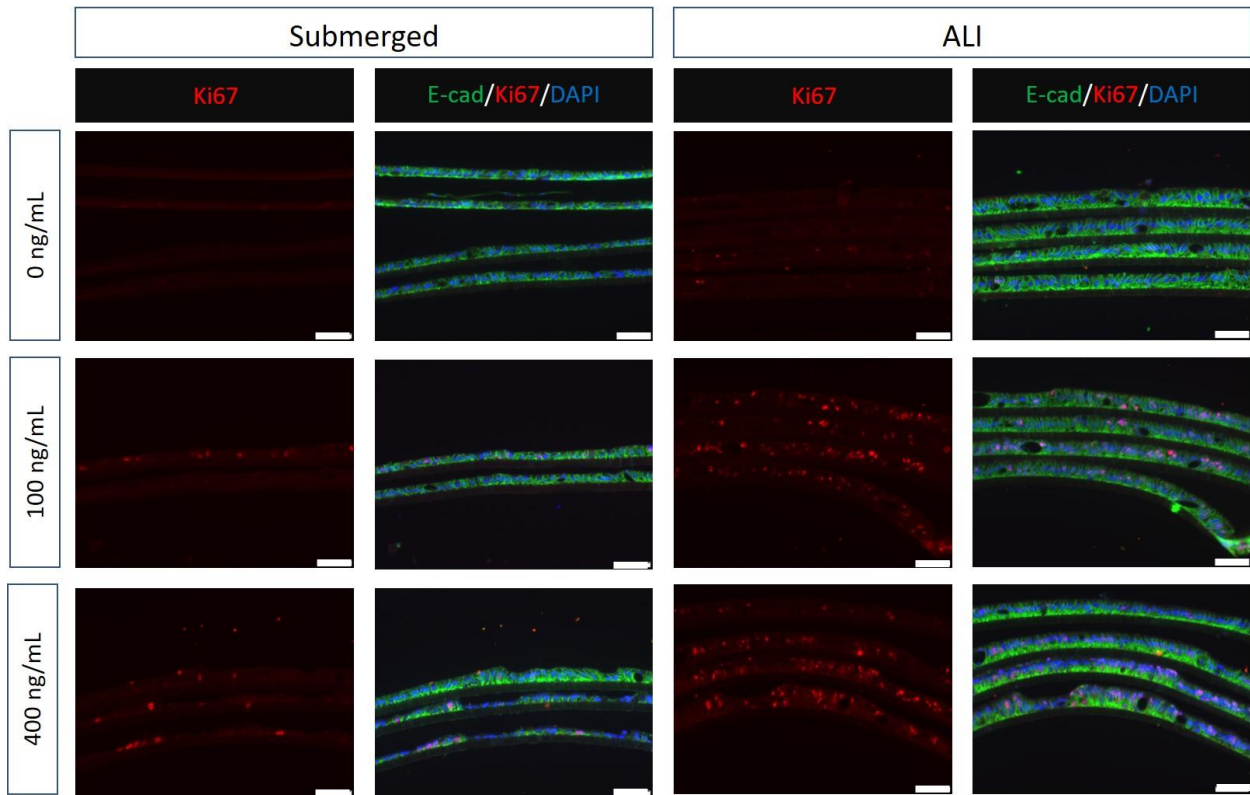
Figure 4-19: Immunofluorescence against ki67 (red) on monolayer cultured with various concentration of Noggin. It showed that as the concentration of Noggin increased, ki67+ cells increased. Scale bar: 50 μm
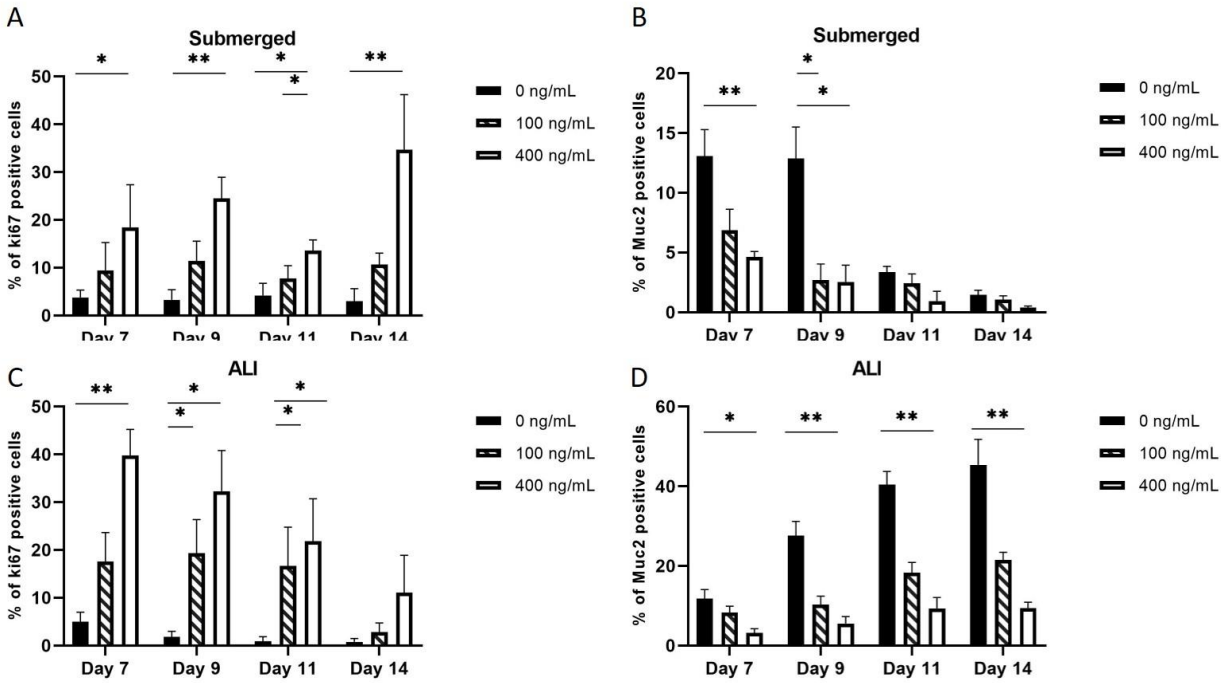
Figure 4-20: Quantitative analysis of ki67 and Muc2 positive cells on monolayer cultured with

various concentration of Noggin. It showed that as the concentration of Noggin increased,

ki67+ cells increased. (n=3)

From H&E results, a significant difference in monolayer morphology was observed (Fig. 4-21). The monolayer was more organized and able to maintain its integrity for up to 14 days when treated with BMP4 under submerged culturing condition.

TUNEL assay showed the decrease in apoptotic cells when the monolayer was treated with BMP4 (Fig. 4-22), corresponding with the result that the apoptotic cells were significantly decreased in co-culture model.

On the other hand, there were more ki67 positive cells in the monolayer without BMP4 treatment which correlate with the results where the Noggin concentration was varied (Fig. 4-23). It also suggesting that with BMP4 treatment, the cells were more quiescent.

From the immunostaining results, there seems to have certain level of effect in the differentiation as the presence of the Muc2 positive cells can be seen (arrow) (Fig. 4-24). However, it did not seem to have significant difference but it did indicate that the presence of the differentiation cue provided by BMP signaling pathway.

To further investigate the exact mechanism involving in the influence of ISEMF on monolayer, the RNA expression level of BMP4 and Cdkn1a was examines. Cdkn1a is known to be a cell cycle arrest gene with certain level of apoptosis inhibition and it is reported that BMP4 could induce the expression of Cdkn1a [21]. It would be informative to examine if there is any correlation between the decrease in apoptotic cells in co-culture system and the expression of Cdkn1a.

From the RT-PCR result (Fig. 4-25), when looking at the monolayer cultured under submerged condition, although expression level overall is low, co-culture system exhibit significantly higher expression level than mono-culture system on day 7 and day 14. Additionally, under ALI culture, not only the expression level overall is significantly elevated, the expression of co-cultured system is significantly higher than mono-culture system most of the time through the experiment.
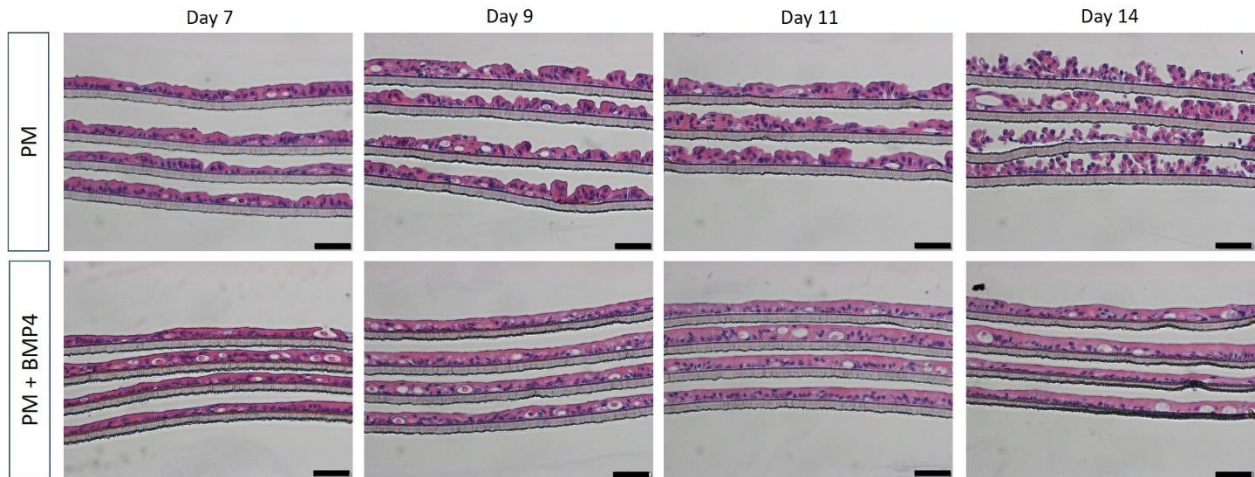
Figure 4-21: H&E histology showed difference in the monolayer morphology between the one with or without BMP4 treatment under submerged culturing condition. The monolayer underwent BMP4 treatment exhibit a more organized morphology. Scale bar: 50 μm

PM		PM + BMP4	
Day 7	Day 11	Day 7	Day 11
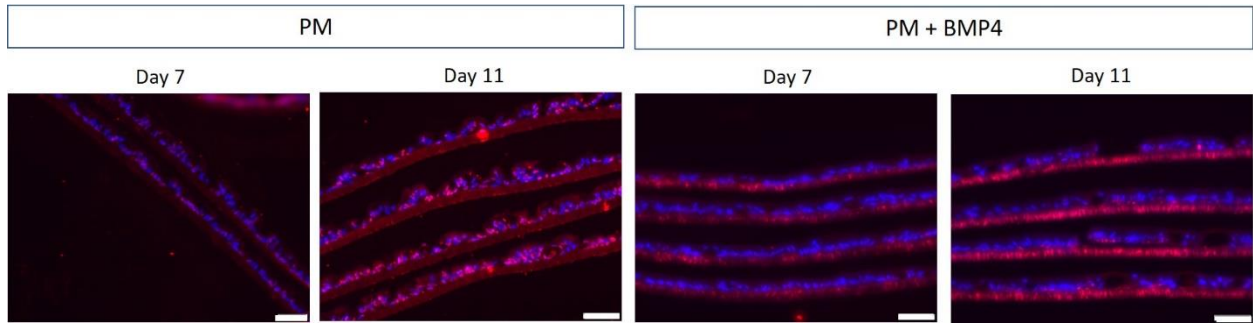
Figure 4-22: TUNEL assay on two monolayers with or without BMP4 treatment. After BMP4

treatment, the decreased in cellular apoptosis could be observed. Scale bar: 50 µm
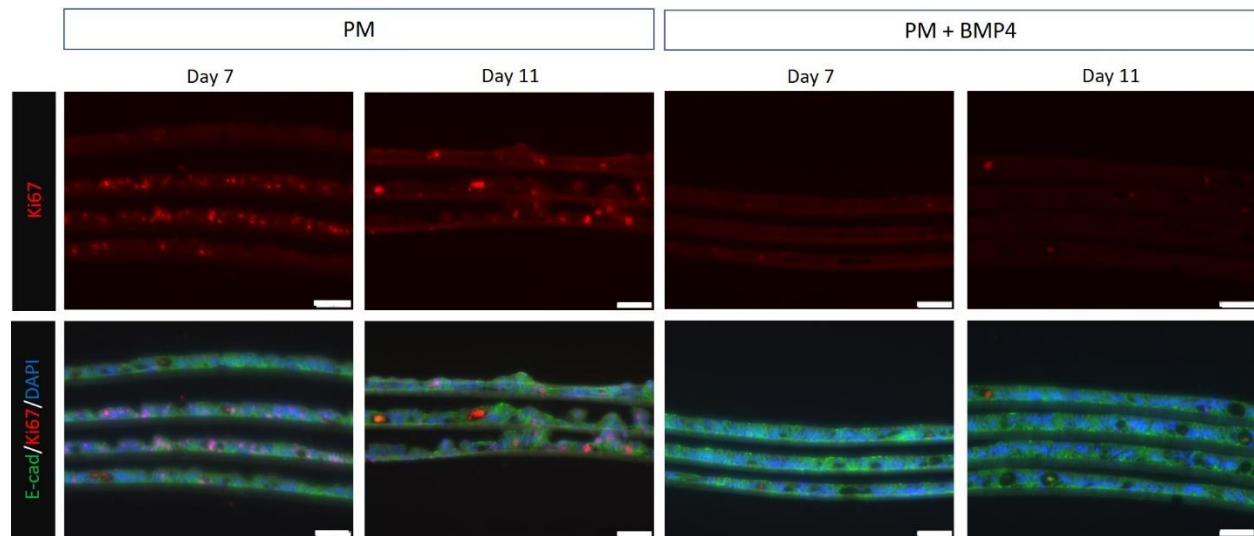
Figure 4-23: Immunofluorescence of the monolayers with or without BMP4 treatment. There were more ki67 positive cells on the monolayers without BMP4 treatment. Scale bar: 50 μm
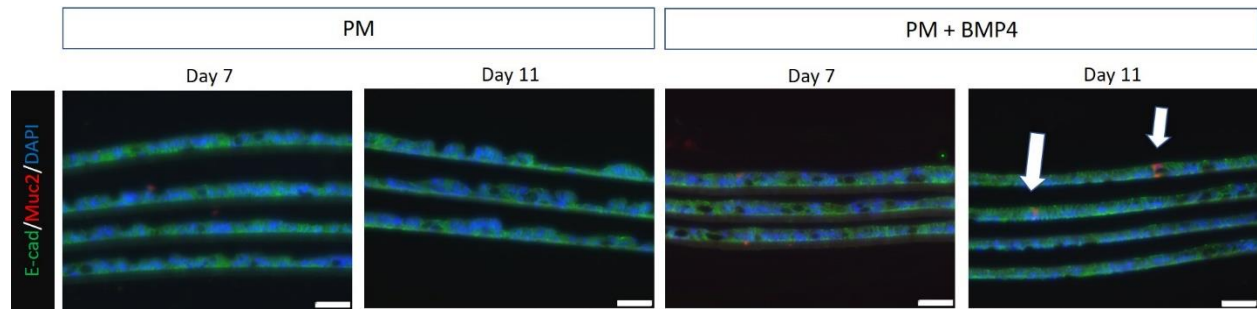
Figure 4-24: Immunofluorescence of the monolayers with or without BMP4 treatment. There were Muc2 positive cells (arrow) present on the monolayers with BMP4 treatment. Scale bar: 50 µm
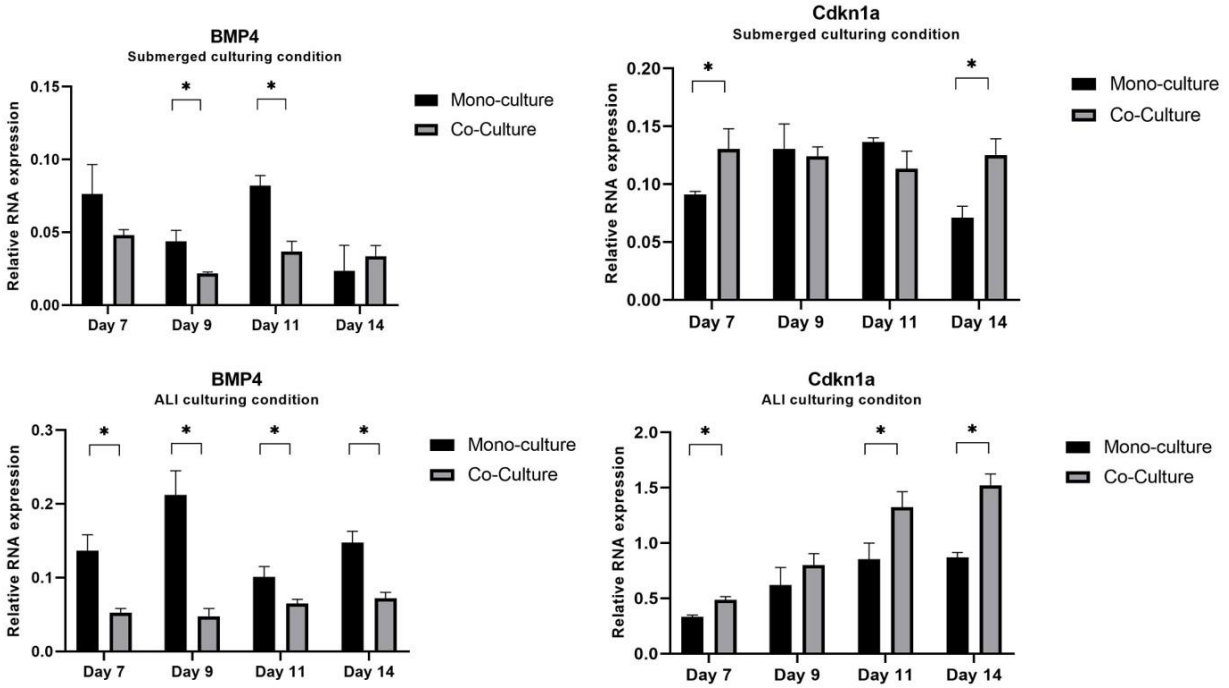
Figure 4-25: RT-qPCR analysis on BMP4 and Cdkn1a gene expression on mono-cultured and co-cultured monolayer. Mono-cultured monolayer had higher BMP4 expression than Co-cultured monolayer. Co-cultured monolayer exhibit higher level expression of Cdkn1a than mono-cultured monolayer. (* < 0.05) (n=3)

On the other hand, the expression of BMP4 is the opposite than what was expected where the expression of mono-culture system was significantly higher than the co-culture system throughout the experiment.

4.4 Discussion

Subepithelial intestinal myofibroblast (ISEMF) has been widely reported to be an important part of the intestinal stem cell niche [5], [7]–[10], it is responsible to coordinate the signals to and from intestinal epithelium. In this study, we highlighted the important role ISEMF played in intestinal monolayer development and shed lights on some of the mechanisms ISEMF employed to achieve such purpose.

From the co-culture platform where ISEMF and epithelium monolayer were growing on the opposite side of the Transwell membrane, we observed the positive effect of ISEMF on monolayer development where monolayer was able to maintain its integrity for up to 14 days with organized morphology. This had not been able to achieve with mono-culture platform. Through ki67/TUNEL analysis, we discover that the percentage of the apoptotic cells was dramatically decreased. The apoptotic cells in the monolayer that was under submerged condition decrease to the level that is comparable to the human intestinal epithelium. This is at least two-fold decrease comparing to the monolayer that was mono-cultured. It is believed that through this decrease in the apoptotic events, the balance between proliferation and apoptosis could be maintain, hence the well-maintained monolayer.

In the differentiation perspective, ISEMF also induced monolayer differentiation where there was an increase in Muc2 positive cells which we have failed to observe in the mono-culture system. At the same time, it seems that the distance between ISEMF and the epithelial monolayer is a factor as well. When there is a distance (i.e. 3mm) between the two cell lines, although the effect of monolayer morphology could still be seen, the effect on differentiation seems to be lost. On

one hand, it is possible the differentiation cue ISEMF provides to the monolayer is short ranged paracrine signal, the two cell types need to be in proximity to communicate. On the other hand, it is also possible the fail to see the effect on differentiation is due to the concentration of the mesenchyme factors responsible for cellular differentiation secreted by ISEMF is too low to be received by the monolayer via diffusion through long distance. From RNA expression analysis, we again see the difference between ALI cultured monolayer and submerged culture monolayer. The expression in the monolayer under ALI cultured monolayer is consistently higher than the one under submerged cultured. The only difference between mono-culture and co-culture is that the expression level of Muc2 is significantly higher than the mono-cultured monolayer. This result indicated that ISEMF was able to provide the differentiation cue for goblet cell (Muc2 positive) differentiation and the effect was able to be enhanced through ALI culturing condition.

To investigate the mechanism ISEMF employed to have such an effect on monolayer development, a simplified culturing system with the same effect of co-cultured with ISEMF is needed, thus culturing monolayer with ISEMF conditioned media was used. With such system, it would be easier to isolate different factors that might be having an effect on the monolayer. The activities of BMP signaling pathway is studied since it was known that BMP signaling pathway inhibit cellular proliferation and promote cellular differentiation [14], [15], [22]–[24] and gene encoded for BMP4 protein is upregulated in ISEMF [13]. To induce BMP signaling at different level, various concentration of recombinant Noggin was added into the culture, and there was dosage related response in Muc2 and ki67 expression. The increase in Muc2 expression and decrease in ki67 expression as the concentration of Noggin decrease, it is indicated that the monolayer was experiencing increased cellular differentiation as BMP signaling became more active. This experiment confirmed the involvement of BMP signaling pathway in co-culture system. On the other hand, an interesting phenomenon was also observed. The monolayer morphology seemed to also be affected by varying concentration of Noggin, especially in the

monolayer cultured without any addition of Noggin, when cultured under ALI condition while there is no significant effect in monolayer morphology when culturing under submerged condition. The ALI cultured monolayer exhibit less uniform and less organized morphology as we decrease the concentration of Noggin. This is likely due to the fact that ki67 positive cells were significant less when there was no recombinant Noggin added in ALI culture comparing to submerged culture, rendering not enough proliferative cells to compensate the loss of apoptotic cells. This phenomenon was also presented on day 14 of monolayers cultured with 100 ng/mL of Noggin between ALI and submerged culturing condition, therefore, the less organized monolayers was observed in later time point in this group.

Since certain level of BMP signaling pathway activity could help maintaining the integrity of the monolayer, recombinant BMP4 was added into the monolayer cultured with proliferation media attempting to induce BMP signaling activity and examine if the same phenomenon could be observed. Interestingly, with BMP4 treatment, monolayer exhibit a much more organized morphology. However, this positive effect was only limited to the monolayer under submerged culture, the monolayer under ALI condition demonstrated a much more disorganized morphology. If looking at this result and the difference in monolayer morphology under ALI condition with varying Noggin concentration, it seems that there exists an optimal level for BMP signaling activity. In terms of differentiation, we did see some level of effects with the presence of Muc2 positive cells when the monolayer was BMP treated, however, due to the loss of integrity of ALI cultured monolayer, the enhancing effect on differentiation cue was lost as well. Further investigation varying different concentration of recombinant BMP4 is required. Additionally, it is also reported that ISEMF is an important source of prostaglandin 2 (PGE2) and PGE2 has an effect in enhancing the expression of Muc2 [25], [26], it is likely that not only BMP signaling is involved in the maintenance and differentiation of the monolayer, but PGE2 as well.

In efforts to identify the key players involved in the effect of co-culture system, the RNA expression level of BMP4 and Cdkn1a was examined. The increased Cdkn1a expression in co-culture system seems to correlate with the decrease of apoptotic cells compared to mono-culture system with Cdkn1a being cell cycle arrest gene. However, the expression of BMP4 is rather perplexed since it was reported that BMP4 can induce the expression of Cdkn1a [21]. The relationship seemed to be lost here. For further investigation, several other candidates should be examined since there are several other factors also involved in BMP pathway such as BMP2, samd1/5/8 complex.

4.5 Conclusion

This study highlighted the effect of ISEMF on monolayer development through co-culturing the ISEMF with intestinal monolayer. It is well known that ISEMF has supportive effect on stem cell proliferation, however, our study showed that it also has an effect on cellular differentiation at the same time. Furthermore, we also showed that the mechanism of this effect comes from the activity of BMP signaling, albeit the level of activity requires a delicate balance. Lastly, this study underlines the important role the ISEMF played as part of the intestinal stem cell niche.

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CHAPTER FIVE

CONCLUSION AND FUTURE DIRECTION

5.1 List of Novel Findings

The Investigation of Development of the Intestinal Epithelial Monolayer In Vitro

- The existence of innate variabilities among different enterocyte lines which would be significantly manifested in the development in the monolayer

- The balance between proliferative and apoptotic cells is important in monolayer development

The Effect of Air-Liquid Interface (ALI) Culturing Environment on the Development of Human Intestinal Monolayer

- Air-Liquid Interface culturing environment has positive effect on maintaining the monolayer integrity by decreasing the number of cells undergoing apoptosis.

- The morphology and polarity of the epithelial cells cultured under ALI environment closely resemble the matured enterocytes in human intestine.

- The epithelial cells are more differentiated under ALI culturing condition.

- ALI serves as signal amplifier where it can enhance the existing differentiation signal but could not direct the cells to take on different cell lineage.

The Interaction between Intestinal Epithelial Monolayer Development and Intestinal Subepithelial Myofibroblast (ISEMF)

- Through co-culture with ISEMF, the positive effect on monolayer development was demonstrated. The number of apoptotic cells was dramatically decreased when co-cultured with ISEMF, thus the integrity of the monolayer is able to be maintained.

- ISEMF also provided differentiation cue for goblet cell differentiation and such cue can be further enhanced through ALI culturing condition to increase Muc2 expression.

- Bone Morphogenetic Protein (BMP) signaling pathway plays a role in the effect observed on the development and differentiation of the monolayer.

5.2 Conclusions and Future Directions

This study highlighted the importance of maintaining the balance between cellular proliferation and cellular differentiation and apoptosis during intestinal monolayer development. In vivo, the interactions and communication among different cell types, multiple signaling pathways via growth factors, cytokine and mesenchyme signals in the stem cell niche shouldered this responsibility in keeping cellular growth and development in check. This balance is especially important in monolayer development in vitro. With well-defined growth area (i.e. the surface area of the Transwell membrane) and the interplay among different factors involving in maintaining balance in vivo not yet fully deciphered, maintaining the balance between two sided of the scale, one being proliferation and apoptosis being the other, proved to be a sensitive and tortuous task.

From our investigation, the variabilities among different enterocyte lines in monolayer generation we observed would potentially affecting the accuracy of the experiments employed intestinal epithelial monolayer platform. In the attempt to identify the underlying factor resulting in such variabilities, although it is not conclusive, the balance between ki67 positive cells and apoptotic cells seems to be a factor. After comparing the percentage of proliferative cells and apoptotic cells in the monolayers generated from two different enterocyte lines, the one exhibiting homogeneous, organized monolayer seems to possess more proliferative cells than the other throughout the experiment. The failure to observe a statistical difference might be due to the variabilities among different repeated yet independent experiments. From our study, the variabilities are not only an innate characteristic among different enterocyte lines, the culturing

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condition might contribute to the variabilities presented in the monolayer development. For in vitro culturing, L-WRN conditioned media was used and played an important part in the in vitro culture system. The L-WRN conditioned media contained three stem cell niche factors, Wnt3a, R-spondin 3 and Noggin, that play major roles in maintain the stem cell proliferation. It is used in lieu of the recombinant factors which are not as cost effective comparing to conditioned media. However, to our experience, the batch to batch variabilities of the L-WRN conditioned media is often observed combining with the fact that the exact concentration of the three growth factors are unknown, this variabilities of the conditioned media would be easily translated and amplified to the development of the monolayer in vitro where maintaining the balance is an intricate task. One proposed method to eliminate this additional variability in effort to truly verified whether the difference in the monolayer morphology we observed lies within the ability of the cells to balance between proliferative cells and apoptotic cells, a culturing system fully utilizing recombinant proteins could be used. With this system, the concentrations of each factors are known, hence eliminating the unknowns comes along with L-WRN conditioned media.

Air-Liquid Interface culturing condition has been widely used in airway epithelial monolayer development in vitro [1]–[4]. Our study demonstrated that it also has the similar effect on intestinal epithelial monolayer development in promoting monolayer integrity and differentiation. Under ALI culturing condition, the epithelial cells recapitulate the physiologically relevant morphology, polarized columnar morphology. It also served as an amplifier capable of enhancing the existing differentiation cue. Furthermore, it is discovered that ALI was able to better maintain the monolayer integrity through decreasing the numbers of cells undergo apoptosis, hence the balance between proliferative cells and apoptotic cells are restored. Although it was reported that the difference in metabolic mechanism the cells take on under two culturing conditions contribute to the effect of ALI culturing conditions on the monolayer development [4]–[6], from our study, it only suggests to be part of the reasons. Further investigation is required. One other possibility

worth looking into is the activity of mitogen activated protein kinase (MAPK) activity. A study done on cow mucosal epithelial cells showed the increase in phosphorylated ERK1/ERK2 in the ALI cultured monolayer, suggesting the activation of MAPK activities [7]. Additionally, the MAPK signaling pathway is known to responsible in cellular proliferation, differentiation and cytoskeletal alteration. One proposed way to examine this hypothesis would be utilizing MAPK inhibitor in the monolayer culturing system and examining any differentiation in cellular morphology and differentiation between two culturing conditions.

Through investigating the interaction between the intestinal epithelial monolayer and the intestinal subepithelial myofibroblast (ISEMF), we have discovered that, through co-culture, it not only yielded a monolayer with better integrity comparing to monolayer culture, the cells are also more differentiated at the same time. The strategy the ISEMF employed to maintain the balance between proliferation and apoptosis is similar, by inhibiting cellular apoptosis. Additionally, the Muc2 expression is significantly increased under ALI culturing condition suggesting ISEMF also provided differentiation cue for goblet cell differentiation and is able to be enhanced through ALI culturing. The driving force behind the effect of ISEMF on monolayer development is proved to be the activity of BMP signaling pathway, we were able to manipulate the degree of increase and decrease of Muc2 positive cells and ki67 positive cells by varying the concentration of Noggin, an BMP signaling antagonist. Furthermore, through the addition of recombinant BMP4 into proliferation media, the integrity of the monolayer dramatically increased. However, even though with BMP4 treatment, the integrity of the monolayer was better maintained, no significant increase in muc2 expression is observed. This result might suggest that there is other player involved in inducing cellular differentiation, other than BMP signaling activation. One potential candidate would be the expression of prostaglandin 2 (PGE2). It is reposted that not only PGE2 gene is upregulated in the ISEMF, it also induced the Muc2 expression [8]. Additionally, it was also observed that varying concentration of Noggin, the monolayer morphology seems to be affected

when there was no noggin added into the culturing system. It might suggest that the activity of the BMP signaling pathway needed to be kept in check as well.

Additionally, major part of this study is focusing on altering one arm of the scale, cellular differentiation and apoptosis, to maintain the balance. The other arm, cellular proliferation, would be worthwhile investigating as well.

Overall, this study provided an insight in the monolayer development in vitro and the importance in maintaining the balance between cellular proliferation and cellular apoptosis. It is not suggested that which culturing technique is optimal, depending on the need of the experiment, some might find media switching from proliferation media to differentiation media is sufficient since they are only looking into short term effects. However, this study provided some tricks that could tip the scale back to balance if long term observation is required. We also demonstrated a technique to generate a monolayer that is physiologically relevant in cellular morphology and possess both proliferative cells and differentiated cells. We also have provided more insights as to the strategies non-epithelial cells, as part of the stem cell niche, utilized to maintain intestinal epithelium homeostasis in vivo.

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