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TREBALL DE FI DE GRAU

BIOMIMETIC INTESTINAL MUCOSA MODEL FOR EPITHELIAL MIGRATION STUDIES

Clàudia Monné Tomàs

Tutor acadèmic: Anna Ardèvol Grau

Tutor de pràctiques: Vanesa Fernández Majada

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Index

Institute data 2
Abstract 3
Introduction 4
1. Anatomy and biology of the small intestine 4
2. Intestinal subepithelial myofibroblasts (ISEMF) 7
3. Organoids as an *in vitro* model for small intestine epithelium 8
Hypothesis and objectives 11
Materials and methods 12
1. Hydrogel fabrication 12
2. PDMS fabrication 13
3. Fibroblasts cell culture 13
4. Intestinal Crypt Isolation and Culture 13
5. Intestinal Subepithelial Myofibroblasts (ISEMF) Isolation and Culture 15
6. Mitomycin C treatment 16
7. Cell culture on hydrogels 16
8. Epithelial cells and fibroblast co-culture on hydrogels 16
9. Immunostaining 17
10. Fibronectin and collagen quantification 17
11. Epithelial monolayer coverage quantification 18
12. ISEMFs labelling with PKH26 red fluorescent cell linker 18
13. Preparation of the sample for the life imaging migration experiments 18
14. Life imaging 19
Results and discussion 20
1. Experimental setup 20
2. Hydrogel fabrication 21
3. NIH-3T3 fibroblast monolayer characterization 22
4. NIH-3T3 fibroblasts and epithelial cells co-culture characterization 25
5. ISEMFs culture characterization 28
6. ISEMFs and epithelial cells co-culture characterization 29
7. Epithelium migration analysis 32
Conclusions 35
Bibliography 36
Self-assessment 39
Acknowledgements 40

Institute data

The Institute for Bioengineering of Catalonia (IBEC) is an institution engaged in basic and applied research in bioengineering and nanomedicine. The institute was created by the Government of Catalonia, the University of Barcelona and the Polytechnic University of Catalonia in December 2005 and is located at the Barcelona Science Park (Parc Científic de Barcelona). In 2014 IBEC was named a "Severo Ochoa Centre of Excellence" by the Spanish Ministry of Economy and Competitiveness by the Spanish Ministry of Economy and Competitiveness.

In particular, I had been working with the Biomimetic Systems for Cell Engineering group coordinated by the group leader Elena Martinez Fraiz. Their work is based on the fact that *in vitro* assay platforms involving human cells are increasingly important to study tissue development, tissue regeneration, construct models of disease or develop systems for therapeutic screening that predict the human *in vivo* context. But, there is a main conceptual problem of the standard *in vitro* cell-based assays and is, that they rely on two dimensional monolayer cellular cultures, which fail to replicate the complexity of living systems. There is an urgent need to create technological platforms with complex cell culture systems that mimic better the tissue-like cellular microenvironment. But, specifically, in the project I had been working on, they aim to develop functional, *in vitro* models of the intestinal epithelium, which protects the area against physical, chemical and microbial damage. As this is one of the most actively renewing tissues in the body, as well as a major site of carcinogenesis, achieving working models of the intestinal epithelium would be invaluable for basic research into intestinal disease modelling, drug discovery and tissue replacement, as well as providing essential tools for adult stem cell research. In order to achieve this objective, they combine microfabrication techniques, tissue engineering components and recent advances in intestinal stem cell research, exploiting stem cell self-organizing characteristics to recreate the 3D morphology, spatio-chemical gradients and dynamic microenvironment of the living tissue. The achievement of such models will be a step beyond state-of-the-art organoid models, helping boost our understanding of cell physiology, adult stem cell behaviour and organ development, as well as opening up new areas of research on human intestinal diseases.

Abstract

Three layers compose the intestinal mucosa of aldult mamals: the epithelium, the lamina propia and the *muscularis mucosae*. The intestinal epithelium is the fastest self-renewing tissue in the body. This process involves the migration of cells from the base of the intestinal crypts, where the intestinal stem cells are located, up to the tip of the villi structures. Underneath the epithelium, we find subepithelial myofibroblasts (ISEMF) and they have been frequently associated with regulation of intestinal epithelium renewing even the mechanism remains poorly understood. We hypothesize that ISEMF do not have only a structural function under the intestinal epithelium but they facilitates epithelial expansion and migration. For this reason, we performed a new intestinal mucosa *in vitro* model composed by a layer of myofibroblasts and a layer of epithelial cells cultured on a hydrogel, mimicking the native environment. After characterize this *in vitro* model we used it for migration studies and we could observe an intimate mechanical interaction between epithelial cells and myofibroblasts.

Key words:

Intestinal epithelial cells, intestinal organoids, intestinal subepithelial myofibroblasts, epithelium migration, hydrogel, biomimetic model.

Introduction

1. Anatomy and biology of the small intestine

The small intestine is the part of the gastrointestinal tract between the stomach and the large intestine. The human small intestine dimension are approximately 7 meters in length and about 3 cm of circular diameter. It is further divided into three sections: the duodenum, the jejunum, and the ileum. The main function of the small intestine is the absorption of nutrients and minerals but also constitutes the largest and most important protective barrier against the external environmental insults. The intestinal epithelial barrier is essential for maintaining tissue homeostasis because of its ability to prevent invasion of microbes and microbial metabolites inside the body. Anatomically, the intestinal epithelium is formed by projections called villi, which increase the nutrient absorption surface, and invaginations into the underlying connective tissue, named crypts of Lieberkühn. Villus and crypts are lined by a monolayer of polarized epithelial cells that have membrane protrusions in the apical side called microvilli, adult intestinal stem cells and their progeny are localized at the bottom of the crypts. The organization of the epithelium into crypts and villus is shown in *Figure 1*. At the histological level, the small intestine has four different layers: mucosa, submucosa, *muscularis* and serosa (Figure 2). The mucosa is the innermost tissue layer and is in direct contact with the lumen. Structurally and functionally, it is the most complex and important area. The submucosa is the layer of dense irregular and connective tissue that supports the mucosa as well as joins the mucosa to the bulk of underlying smooth muscle. The

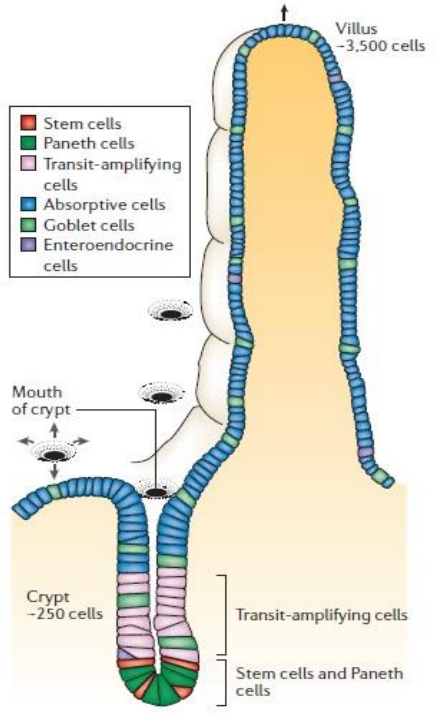

Figure 1. The distribution of epithelial cell types in the mammalian small intestine. *A villus with one of the crypts that contribute to renewal of its epithelium. Arrows indicate the upwards flow of cells out of the crypts. Stem cells lie near the crypt base. It is uncertain whether they are mixed with, or just above, the Paneth cells. Above the stem cells are transit-amplifying cells (dividing progenitors, some of them already partially differentiated); and above these, in the neck of the crypt and on the villus, lie post-mitotic differentiated cells (absorptive cells, goblet cells and enteroendocrine cells. (Crosnier, Stamataki, & Lewis, 2006)*

muscularis is a region of muscle adjacent to the submucosa membrane. It is responsible for gut movement, or peristalsis. Finally, the serosa is the outermost layer of the intestine. It is a smooth membrane consisting of a thin layer of cells that secrete serous fluid, and a thin layer of connective tissue.

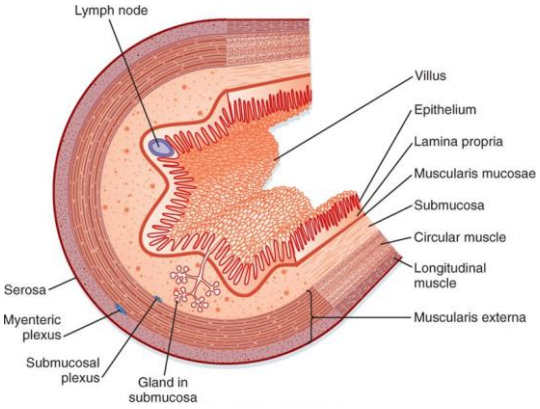

Figure 2. Layers composing the wall of the GI tract. *The mucosa is the innermost layer. It consists of the epithelium, the lamina propria, and the muscularis mucosae. The next layer is the submucosa. The submucosa consists largely of loose connective tissue. The muscularis externa or muscularis propria typically consists of two substantial layers of smooth muscle cells: an inner circular layer and an outer longitudinal layer. (koppen & Stanton: Berne and Levy Physiology, 6th Edithion. Copyright © 2008 by Mosby, an imprint of Elservier, Inc. aAl rights reserved)*

Three layers compose the intestinal mucosa: epithelium, lamina propria and *muscularis mucosae*. The epithelium is the first layer facing the intestinal lumen and it is composed of epithelial cells, which are disposed as a single layer of polarized cells attached to a base membrane composed of extracellular matrix (ECM) proteins. The second layer, the lamina propria, consists of subepithelial connective tissue containing myofibroblasts, blood vessels, nerves and several lymph nodes. The third and deepest layer called muscularis mucosae. This is a continuous sheet of smooth muscle cells that aids in the action of continued peristalsis along the gut.

The intestinal epithelium is the fastest self–renewing tissue in the adult mammals. This high tissue renewal is sustained by the high proliferative intestinal stem cells (ISCs) which reside at the bottom of the crypts. Intestinal stem cells divide and give rise to transit-amplifying cells, which spend approximately two days in the crypt, where divide 4–5 times before they terminally differentiate into the specialized intestinal epithelial cell types while migrate up to the villi tip (van der Flier and Clevers, 2009). Three types of differentiated specialized epithelial cells cover the villi: the absorptive enterocytes, mucous-secreting goblet cells, and hormone-secreting enteroendocrine cells. Three days after their terminal differentiation, the cells reach the tip of the villus, undergo spontaneous apoptosis and are shed into the gut lumen. Another type of intestinal cells are the Paneth cells, they are unusual in that they settle at the crypt bottoms and

represent the only differentiated cells that escape the upward migration (*Figure 1*) (Crosnier, Stamataki and Lewis, 2006).

It was thought for decades that the stem cells localized at the bottom of the crypts, also known as crypt-base columnar cells (CBCs) were the only intestinal pluripotent stem cells that maintained the proliferative activity of crypts. Further studies have demonstrated that other populations of stem cells located outside of the crypt base, specifically cells in the +4 position of the crypt, can revert into multipotent stem cells upon intestinal damage. These findings demonstrate the plasticity of the stem and progenitor cells in the intestinal tissue is necessary to maintain homeostasis in the harsh environment of the intestinal lumen (Buczacki *et al.*, 2013). The identification of specific markers for each of the stem cell populations in the intestine has been a topic of great interest during the last decade. Barker et al. defined Lgr5 as a stem cell marker of the crypt-base columnar cells (CBCs). These authors employed a Lgr*5-EGFP-ires-CreER* transgenic mice together with a Rosa26-lacZ reporter strain and perform lineage-tracing experiments in the intestine. Their results clearly showed that Lgr5+ cells are located in the bottom of the crypts, coinciding with the CBCs and possess stemness, long-term self-renewal ability, and multipotent differentiation capability (Barker *et al.*, 2007). Lgr5 is a co-receptor for the canonical Wnt pathway, which is activated through the binding of R-spondin proteins. Wnt signalling has been shown to be essential for intestinal stem cells (ISCs) maintenance, and its removal results in terminal differentiation and ultimate loss of the intestinal epithelium (Date and Sato, 2015).

It has been shown that Paneth cells, which are tightly nested between ISCs in the crypt base, are very important elements of the intestinal stem cell niche. Paneth cells are secretory cells that on the one hand produce antimicrobial defences that keep the environment surrounding the ISCs sterile and on the other hand secrete supportive growth factors, such as Wnt3a and R-spondin, to keep the identity and boost the proliferation of the ISCs. It has been reported that Paneth cell-derived Wnt3 is indispensable for *in vitro* intestinal epithelial cultures (Sato, Van Es, *et al.*, 2011). However, ISC niche maintenance *in vivo* is preserved even with conditional epithelial Wnt3 deletion and Paneth cell ablation. This suggests the presence of redundant sources of Wnt signalling from surrounding non-epithelial cells within the niche (Lei *et al.*, 2014).

2. Intestinal subepithelial myofibroblasts (ISEMF)

Myofibroblasts have been described as intermediate cells between fibroblasts and smooth muscle cells. Besides of the typical fibroblast characteristics, such as abundant endoplasmic reticulum and extracellular matrix protein secretion, myofibroblast also exhibit properties of smooth muscle cells, including bundles of myofilaments and gap junctions which provides these cells with contractile capacity (Chaponnier and Gabbiani, 2005). Myofibroblasts were firstly discovered in granulation tissue associated with skin wound healing (Gabbiani, Ryan and Majno, 1971). Their enhanced collagen secretion and subsequent contraction are considered part of the normal wound healing response and crucial to restore tissues integrity. It has been observed that in response to tissue injury fibroblasts transition to contractile myofibroblasts in order to remodelling and repair the damaged tissue. This is a stepwise process in which fibroblasts first evolve into protomyofibroblasts by expressing stress fibers containing β- and γ-cytoplasmic actins and then fully differentiate into myofibroblasts that are characteristic to express α-smooth muscle actin (α-SMA) (Chaponnier and Gabbiani, 2005). Once tissue is repaired myofibroblasts are cleared by apoptosis or become de-activated. Otherwise, persistent myofibroblasts activities cause accumulation and contraction of collagenous ECM, a condition called fibrosis. Thus, is typical to find myofibroblast in all fibrotic disease and as well as in some epithelial tumours (Hinz *et al.*, 2007).

Besides of the presence of myofibroblasts in the before mentioned pathological conditions, can also be found as a resident cell in several organs under physiological conditions. For instance, myofibroblasts are present in the lung alveolar septa and in the intestinal lamina propria. (Hinz, 2015). The myofibroblasts from the intestinal lamina propria are known as intestinal subepithelial myofibroblasts (ISEMF). ISEMF are located underneath the intestinal epithelial cells. These fibroblasts form a basket-like network around the crypts and villi that are tightly arranged in the crypt and lower part of the villi but their morphology changes towards the upper part where they assume a stellar shape, suggestive of a different function depending in which area of the axis crypt-villus are found. Intestinal myofibroblasts are recognized as cells expressing α-SMA along with vimentin but negative for the smooth muscle cell marker desmin (Bochaton-Piallat, Gabbiani and Hinz, 2016).

ISEMFs have been associated with the regulation of intestinal epithelium at different levels. On the one hand it is thought that ISEMF might constitute an important source of factors that favours stem cells growth, including Wnt2b, Wnt4, Wnt-5a and Wnt-5b (Farin, Van Es and Clevers, 2012) and the enhancer of the Wnt pathway R-spondin 2 (Kim KA *et al.* 2008). *In vitro* studies have demonstrated that ISEMFs serve as natural feeder cells for *in vitro* intestinal epithelial culture, as long as ISEMF-conditioned medium favours intestinal enteroids growth an viability for long culture periods (Lei *et al.*, 2014). In addition, taking in consideration the particular contractile characteristics of ISEMFs, other supportive roles have been suggested. However, the mechanism of their interaction with overlying epithelium remains poorly understood.

3. Organoids as an *in vitro* model for small intestine epithelium

There has always been a great interest in culturing primary intestinal epithelial cells (IEC) *in vitro*. For instance, in the early nineties Evans et al. devolved a method to culture rat small intestine cells seeded two-dimensionally in culture dishes and tested the effects of several grow factors and substrates in the intestinal epithelial cells growth. However this protocol only allowed IECs to grow for around 10-14 days, indicating technical difficulties in maintaining long-term survival and proliferation of IECs *in vitro* (Evans *et al.*, 1992).

The first report of culture system that could promote long-term survival of intestinal stem cells consisted on culturing intestinal fragments containing epithelial and mesenchymal cells from neonatal mice (Ootani *et al.*, 2010). They employed an air-liquid interface model that, when supplemented with fetal bovine serum, produced cyst-like structures containing all major cells types of the adult mouse intestine that could be maintained for over one year. In parallel, Dr. Hans Clevers' group published a technique for culturing intestinal epithelial cells in a 3D culture system (Sato, Stange, *et al.*, 2011). They sorted single Lgr5+ stem cells from the adult mouse intestine and cultured them embedded in a semi-solid, laminin/collagen Matrigel semisphere with a culture medium that mimicked the factors present in the *in vivo* stem cell niche (*Figure 3a*). With these culture conditions, Lgr5+ stem cells were able to give rise to an intestinal organoid after 10-12 days in culture (Figure 3b). Intestinal organoids, also known as

"miniguts" for the structural similarities to the *in vivo* organs, are spherical hollow units with a central space corresponding to the intestinal lumen surrounded by "buds" that represent the intestinal crypts, and villus-like domains connecting the buds. Similarly, to the *in vivo* intestine, in the intestinal organoids Lgr5+ stem cells are intercalated with Paneth cells and located at the crypt base, where divide to generate a pool of proliferative cells that localize at the upper part of the crypt and terminally differentiate cells (enterocytes, enteroendocrine cells or goblet cells) located at the villi-like domains of the organoids (*Figure 3b*). The terminally differentiated cells are ultimately extruded into the lumen from the villus-like domain, mimicking the physiological turnover of the adult intestinal epithelium. The organoids cultured in this manner can also be maintained indefinitely with periodic cell dissociation and passaging (Sato *et al.*, 2009). Specific modifications of the culture medium composition allowed, also the long-term culture of intestinal organoids derived from other regions of the mouse intestinal tract and from other species including humans (Sato, Stange, *et al.*, 2011). The intestinal organoid culture represented the establishment of a novel model system that incorporates many physiological properties of *in vivo* mouse models with the ease-to-culture and ability to manipulate specific variables in the cellular environment that are typical of *in vitro* cell culture models.

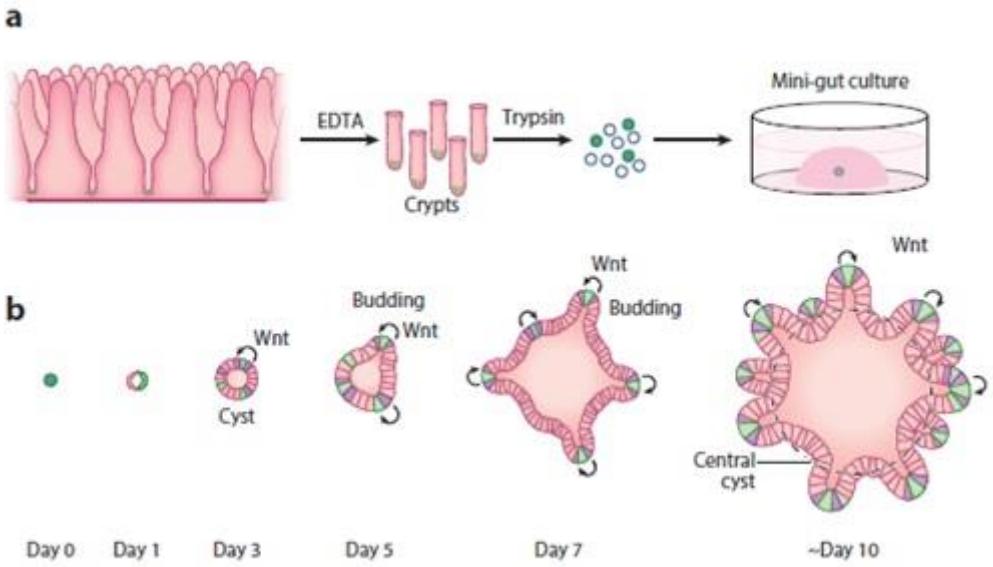

***Figure 3**. Organoid culture. (**a**) Small intestine crypts are isolated and tripsinized into single cells. Single cells (some of them intestinal single cells (ISCs) are embedded into Matrigel with culture medium containing EGF, Noggin, and R-spondin. (**b**) Single ISCs form cyst structures during days 1–4 after single cell culture. After day 5, the cyst initiates bud formation and forms an organoid structure. (Date & Sato, 2015).*

Since the technology to culture primary intestinal epithelial cells as intestinal organoids was stablished, there has been a growing interest in using these culture system for stem cell biology, disease modelling and drug screening studies, as well as a promising source for regenerative medicine assays (Leushacke and Barker, 2014). Organoids had been important in basic biology studies. They lead to a more definitive understanding of stem cell dynamics within the intestinal crypt. For instance, the identification of the complex role of Paneth cells in stem cell maintenance (Sato, Van Es, *et al.*, 2011). Moreover, since organoids can be generated from human patients biopsies, they can be a valuable tool for human disease studies. Patient-derived organoids can be used to study cellular mechanisms behind intestinal diseases (e.g. Crohn's disease, ulcerative colitis, coeliac disease). Organoids also offer an opportunity for large-scale drug screening. Organoid-based screening can either be complementary to 2-dimensional screens, or serve to replace these cell line-based assays with a more physiologically relevant model system (Ranga, Gjorevski and Lutolf, 2014). Finally, in future regenerative medicine, one possible approach would be to place organoid cells in a highly engineered environment with various types of 3D scaffolds to assemble fully functional tissues that precisely recapitulate their original structures. (Nakamura and Sato, 2018).

Despite the significant advantages that the intestinal organoids technology has contributed to the intestinal biology field of research, the use of this culture system has some limitations. The inwards orientation of the organoid makes that the apical side of the epithelium is facing the interior of the organoid, being very difficult to access it from the exterior. Thus, this typical spherical configuration with an inaccessible apical epithelium site hampers any experiments involving drug-stimulation, molecule absorption or bacteria-epithelium interaction, among others. Considering this, finding ways to open the intestinal organoids to obtain constructs with exposed apical/luminal compartment are of great interest.

Hypothesis and objectives

We hypothesise that:

- Intestinal subepithelial myofibroblast contribute to the intestinal epithelial expansion and migration.

In order to demonstrate this hypothesis, this project was structured into two main general objectives:

- Generation and characterization of an intestinal mucosa *in vitro* model composed by a feeder layer of primary intestinal subepithelial myofibroblasts and organoid-derived epithelial cells grown on top of a PEGDA-GelMA hydrogel.
- Study the contribution of the intestinal subepithelial myofibroblasts feeder layer on the intestinal epithelial migration.

Materials and methods

1. Hydrogel fabrication

Hydrogels were fabricated by the photopolymerization of poly(ethylene glycol) diacrylated (PEGDA) and methacrylated gelatin (GelMA) molecules under an ultraviolet (UV) irradiation in the presence of the photoiniciator (Irgacure 2959).

Solutions preparation

10% (w/v) PEGDA (Mw: 4000 Da) (Sigma, USA) plus 1%(w/v) 2-hydroxy-1-(4-(hydroxyethoxy)phenyl)-2-methyl-1-propanone (Irgacure 2959; Sigma Aldrich, USA) solution was prepared using Dulbecco's Modified Eagle Medium (DMEM) (Gibco, USA) as a solvent and incubating it at 65ºC under stirring during 2h. Simultaneously, 15% GelMA solution was prepared dissolving lyophilized GelMA in DMEM and incubating it at 65ºC during 2h under stirring. When the solutes were completely dissolved, the two solutions where mixed, by incubating them together for an additional hour at 65ºC under stirring. Equal volumes of both solutions were mixed obtaining a final blend polymer solution of 5% PEGDA+7,5% GelMA+0,5% photoinitiator. Mixed solution was filter using a filter of 0.22µm.

Hydrogel fabrication

As a container to fabricate the hydrogel we employed a PDMS (Polydimethylsiloxane) (Sylgard, UK) chip with a pool of 6 mm of diameter placed on top of a plastic surface. The pool was filled with 50µL of hydrogel solution and then covered by a cover glass (0,19 mm on thickness). Hydrogel solution was photocrosslinked by exposing under UV light (λ= 365nm) at a UV power density of 20,0 mW/cm2 during 154 seconds. Finally, the hydrogels were washed in Phosphate Buffer Saline (PBS; pH=7,4) (Gibco, USA) to remove the non-reactive hydrogel solution and were placed in a 24-well plate. Hydrogels were let to swell in PBS with penicillin-streptomycin 10% (v/v) at 4ºC for 2 days. Hydrogels were kept at 4ºC for not more than one month.

2. PDMS fabrication

The weight of PDMS polymer needed and 10% (w/w) of crosslinker (Sylgard, UK) were calculated. The PDMS polymer and crosslinker were weighed together and mixed using a Pasteur pipette. The solution was placed in a vacuum desiccator until all gas bubbles were removed. Next, the solution was poured into the desired mold, and finally PDMS was cured in a completely horizontal surface at room temperature overnight. For PDMS ring fabrication, PDMS was poured in wells of a 6-well plate, after PDMS curing we employed an 8mm punch to make a whole in the middle of the PDMS disk. For PDMS-stencil preparation PDMS was spined at 500rpm using a Spin Coater to obtain a thin film of PDMS, after curing PDMS was cut using a scalpel to obtain stencils with the desired dimensions (2 cm long and 2mm width).

3. Fibroblasts cell culture

Cells were manipulated under sterile culture hood and maintained in a 95% air-5% CO2 humidified incubator at 37ºC. When cells are 70-80% confluent they were detached from the flask by treating them with Trypsin-EDTA (0,25% w/v)(25200, ThermoFisher Scientific, Spain) and incubating them at 37ºC for 5 minutes. After, the cell suspension was diluted in a DMEM complete medium (Gibco, USA) with 10% fetal bovine serum (FBS) (Gibco, USA), 1% streptomycin-penicillin (Gibco, USA), 1% glutamine (Gibco, USA) and 1% non-essential aminoacids (Gibco, USA). Then, the total volume was centrifuged at 1200rpm for 5 minutes. Supernatant was removed, and the pellet was resuspended with 6mL fresh medium/T25 Flask (TermoFischer Sientific, Spain) and seeded into T25 new flasks. The final flasks number depends on the initial cell concentration. Every 2-3 days, the medium was changed by new fresh medium.

4. Intestinal Crypt Isolation and Culture

Small intestinal crypts were isolated from 17 weeks old *Lgr5-EGFP-IRES-creERT2* adult mouse female. These transgenic mice have GFP expression driven by the endogenous promotor of Lgr5 stem cell marker (Barker *et al.*, 2007).

Mouse small intestine was harvest and placed into a 10 cm petri dish with PBS and all fat and mesentery from the tissue was removed. It was washed by moving the tissue around with a forceps. After, the intestine was cut open longitudinally and the luminal contents were washed away with PBS. The intestine was opened up with tweezers making sure that the mucosal side is facing upwards. The villus were scraped off carefully by using a haemocytometer coverslip. The villus were washed off with PBS and the intestine was chopped into 2-4 mm pieces and transferred into a 50 ml Falcon tube (TermoFischer Sientific, Spain). We wash the intestine pieces by adding 10 ml of PBS. The content was pipetted up and down a few times. After letting the pieces settle, the supernatant was aspirated and more PBS added. This washing step was repeated between 15 and 20 times, until the supernatant was clear. After, 25ml 2mM EDTA buffer (Invitrogen, Spain) in PBS were added and leaved for 20-30 minutes at 4ºC with shaking. After incubation, the small pieces were allowed to settle down and the supernatant was removed. 10 ml PBS with 10% (v/v) FBS was added and mixed with the intestine pieces. The supernatant was collected and passed through a 70 µM strainer (Biologix, USA). This process was repeated 3 more times using new strainers in order to obtain the crypt elution fractions. The crypts were examinated under the microscope. The crypt fractions were spined down at 800 rpm 5min. The pellets were re-suspended with 2-5 ml cold completed organoid medium which consists on: Advanced DMEM/F12 with grow factors B27 (20 µL/mL), N2 (10 µL/mL) and n-acetilcysteine (2,5 µL/mL) and the tube was again centrifuged at 600 rpm for 5 minutes at 4°C. The supernatant was removed and the pellet with the isolated crypts, was resuspended in Matrigel®(Corning, USA), 40µL of Matrigel® plus crypts was pipetted in each well of a 24-well plate and incubated for 5 minutes at 37º for Matrigel®. After allowing the crypts-Matrigel suspension to gelify, completed crypts medium (Advanced DMEM/F12 with grow factors B27 (20 µL/mL), N2 (10 µL/mL) and n-acetilcysteine (2,5 µL/mL) containing also EGF (100 ng/mL), Noggin (100 ng/mL), R-spondin (200 ng/ml), CHIR-99021 (1 µM), Valproic acid (1mM)) was added.

5. Intestinal Subepithelial Myofibroblasts (ISEMF) Isolation and Culture

ISEMFs were isolated from 17 weeks old *Lgr5-EGFP-IRES-creERT2* genotype adult mouse female.

Mouse small intestine was harvest and placed into a 10 cm petri dish with PBS and all fat and mesentery from the tissue was removed. It was washed by moving the tissue around with a forceps. After, the intestine was cut open longitudinally and the luminal contents were washed away with PBS. The intestine was opened up with tweezers making sure that the mucosal side is facing upwards. The villus were scraped off carefully by using a haemocytometer coverslip. The villus were washed off with PBS and the intestine was chopped into 2-4 mm pieces and transferred into a 50 ml falcon tube (TermoFischer Sientific, Spain). We wash the intestine pieces by adding 10 ml of PBS. The content was pipetted up and down a few times. After letting the pieces settle, the supernatant was aspirated and more PBS added. This washing step was repeated between 15 and 20 times, until the supernatant was clear. After, 25ml 2mM EDTA buffer (Invitrogen, Spain) in PBS were added and leaved for 20-30 minutes at 4ºC with shaking. After incubation, the small pieces were allowed to settle down and the supernatant was removed. 10 ml PBS with 10% (v/v) FBS was added and mixed with the intestine pieces. While the supernatant is collected for crypts purification, the left over pieces of intestine were placed into a falcon tube containing 10 mL of EDTA buffer 3mM in PBS at 37°C. The suspension was incubated stirring 10 minutes at 37°C. The supernatant was discarded and the pieces were incubated three times more with 10 mL of EDTA buffer 3mM in PBS at 37°C during 10 minutes. After the supernatant was discarded and the pieces were washed with 50 mL of PBS 10 min at 37°C stirring. The supernatant was discarded and 50 mL of DMEM containing 20% of FBS, collagenase 100U/mL (Sigma C-XXX, 413 U/ mg) and DNase 50U/mL (Sigma 1284932, 2000 U/mg) was added. The suspension was incubated 40 min at 37°C stirring. After, the suspension was aspirated using a 10 mL syringe for 1 minute to improve disaggregation. Then, the suspension was filtered on a 70µm nylon filter to remove tissue debris. ISEMFs obtained after the isolation were cultured in a T25 culture flask (TermoFischer Sientific, Spain) with DMEM complete medium plus Normocin (InvivoGen, USA). After ISEMFs attached and formed colonies, they were subsequently passaged and expanded using standard cell culture techniques.

6. Mitomycin C treatment

When cells achieved 70% of confluence, Mitomycin C (MMC) (10 µg/mL) was added to the culture medium. Cells were incubated with the MMC-containing medium for 1 hour, after this time, the medium was removed, cells were washed twice with PBS and fresh-DMEM complete medium was added. Treated cells were incubated at 37ºC for at least 12 hours before seeding them on the hydrogels Cell culture on hydrogels.

7. Cell culture on hydrogels

When MMC-treated or untreated fibroblasts achieved 70-75% of confluence were trypsinized as explained in section 3 (Fibroblasts cell culture). Trypsinazed cells were diluted in fresh medium and cell density was calculated using a Neubauer Chamber. The volume containing the cells needed for the experiment was centrifuged at 1200rpm for 5 minutes. Supernatant was removed, and the pellet was resuspended with the volume of medium needed for the experiment. 90.000 cell in 20µL of medium were seeded on each hydrogel as a single drop (we employed this seeding technique in order to more accurately control the number of cells per hydrogel seeded). When fibroblasts were attached to the hydrogel (normally 2 hours after seeding), 1ml more of fresh medium supplemented with ascorbic acid (0,28mM) was added in each well of 24-well plate. The culture period is indicated for experiment. Note that the same protocol was use to seed NIH-3T3 fibroblasts and primary fibroblasts on the hydrogels.

8. Epithelial cells and fibroblast co-culture on hydrogels

Full-grown 3D intestinal organoids were digested to individual crypts by mechanical breaking using a 23G needle and enzymatic digestion with TrypLE Express (Thermo Fisher #12605010). Single cells were obtained using a similar protocol but applying a longer enzymatic digestion (5 minutes at 37ºC). Intestinal crypts or single cells were counted and centrifuged 3 minutes at 700 rpm at 4ºC. The supernatant was removed, and the pellet was resuspended with the needed volume of completed organoid medium composed of: Advanced DMEM/F12 with grow factors B27 (20 µL/mL), N2 (10 µL/mL) and n-acetilcysteine (2,5 µL/mL) containing also EGF (100 ng/mL), Noggin (100 ng/mL), R-spondin (200 ng/ml), CHIR-99021 (1 µM), Valproic acid (1mM) and Rock

inhibitor (Y-27632) (10 µM). Approximately 400 crypts or 50,000 cells in 20µL were seeded on each fibroblast-covered hydrogel as single drop. We waited at least 3 hours for crypts or single cells to attached to the fibroblasts and added 1 ml more of completed organoid medium per well.

9. Immunostaining

Cells were fixed by washing the samples with PBS and incubate them with formaldehyde (Sigma Aldrich, USA) during 30 minutes at 4ºC under stirring. After fixation, samples were washed 3 times with PBS for 5 minutes each time and incubated with a permeabilization solution (0,5% Triton (Sigma Aldrich, USA) in PBS) for 30 minutes at room temperature under stirring. Next, samples were incubated with a blocking solution of (0,2% Triton and 3% donkey serum (Merck, USA) in BSA (1%)) during at least 2 hours at room temperature under stirring. Samples were washed 3 times with PBS for 5 minutes and incubated with the indicated primary antibody in primary antibody solution (0,1% BSA, 0,3% Donkey serum and 0,2% Triton in PBS) overnight at 4ºC under stirring. The employed primary antibodies and use concentrations were the following; fibronectin (1:100) (Abcam, Spain), collagen (1:250) (Abcam, Spain), β-catenin (1:200) (Abcam, Spain), α-smooth muscle actin (α-SMA) (1:400) (Abcam, Spain), vimentin (1:40) (Abcam, Spain), desmin (1:200) (Sigma Aldrich, USA) . After primary antibody incubation, Samples were washed 3 times with PBS for 5 minutes and incubated with secondary antibodies in secondary antibody solution (0,1% BSA, 0,3% Donkey serum in PBS) for at least 2 hours at room temperature under stirring. The employed secondary antibodies and use concentrations were the following: AlexaFluor 647 α-rabbit (1:500), AlexaFluor 488 α-Goat (1:500) and 647 α-Rabbit (1:500). We also used phalloidin-rodamine (1:140) for actin staining. Finally, all samples were incubated with DAPI (1:1000) during 20 minutes, washed with PBS and mounted for microscope visualization using Fluoromount ^{G} (Southern Biotech, USA)

10. Fibronectin and collagen quantification

Fibronectin and collagen quantifications were done by analysing the immunostaining images using the ImageJ Fiji analysis tool. The Images were converted to TIFF file format

and the brightness and contrast were adjusted. Then, we define representative regions of interest (ROIs) in all the images. Next, it was calculated the number of pixels of each ROI selected using “Calculate Mean" command.

11. Epithelial monolayer coverage quantification

The epithelial coverage was analysed using the ImageJ Fiji analysis tool. The β-catenin staining was used to analyse the covered areas of the hydrogel. In all the images, the epithelium area was defined by fixing a colour threshold making the images binary. Then, the whole hydrogel area was calculated, and afterwards the covered areas.

12. Intestinal Subepithelial Myofibroblasts (ISEMFs) labelling with PKH26 red fluorescent cell linker

PKH26 is a type of lipophilic dye with long aliphatic tails that incorporates into lipidic regions of the cell membrane and emits red fluorescence. ISEMFs were labelled with the fluorescence dye PKH26 (Sigma-Aldrich, St. Louis, MO, USA) according to the manufacturer’s protocol, but modifying the total volume of the reaction and dye concentrations used to be adjusted to the ISMFs. Briefly, a total of 250.000 ISEMFs were trypsinized using Trypsin-EDTA, washed with serum free DMEM and resuspended in 100µL of Diluent C from the PKH26 Red Fluorecent Linker kit. The cell suspension was mixed with the equal volume of dye solution. Different dye concentrations were tested: 0,5µM, 5µM and 10µM. Immediately after mixing, the solution was incubated 2 minutes at room temperature with periodic shaking. The staining reaction was stopped with 200µL of fetal bovine serum. The cell suspension was washed 3 times with complete DMEM medium and observed using fluorescence microscopy.

13. Preparation of the sample for the life imaging migration experiments

Hydrogels with or without fibroblast seeded on top were placed into a fresh 6-well plate without medium. Then, a PDMS stencil (2 cm long and 2mm width), was carefully placed on each of the hydrogels. Additionally, a PDMS ring was placed around the hydrogel using high-vacuum silicone grease (Dow Corning high-vacuum silicone grease, Sigma-Aldrich, USA) to fix the PDMS stencil in place and reduce the culture cavity. Once the

stencil-on-hydrogel set-up was assembled we seeded the previously stained ISEMF on top of each hydrogel set-up. 60000 cell in 20µL of complete crypt medium were seeded as a drop on each of the hydrogels. After 2 hours, when ISEMF were attached to the hydrogel, 500µl more of fresh complete crypt medium supplemented with ascorbic acid (0,28mM) was added in each well of 6-well plate. One day after, epithelial single cells were seeded on top of the hydrogels-stencil setup with or without fibroblasts, using the protocol described in section 7 (cell culture on hydrogel). After 6 days of epithelial single cells seeding, PDMS stencil were removed. For a better image acquisition of the cells hydrogels were placed them in a 24-well plate with glass bottom (MatTek Corporation, USA). Samples were fixed to the glass bottom of the plate with two-faced PSA adhesive small pieces to avoid displacement of the hydrogels during the video reordering. Completed organoid medium was added in each well. PDMS-stencil and PDMS ring were obtained as explained in section 2 (PDMS fabrication).

14. Life imaging

Life image experiments were conducted at 10x magnification with Axio Observer 7 epifluorescence inverted microscope (Zeiss) with temperature (37 °C), humidity and CO_2 (5%) regulation. Phase contrast and Alexa 546 channels were used and images were acquired every 10 minutes during 15 hours.

Results and discussion

1. Experimental setup

The main goal of this project was to generate an *in vitro* intestinal epithelial mucosa model to study the potential contribution of the intestinal epithelial myofibroblasts to the expansion and migration of the intestinal epithelial cells.

With this aim in mind, we designed the following experimental setup to obtain the desired culture model. Our objective was to mimic the two innermost mucosa layers, the intestinal epithelium and the lamina propria. The intestinal epithelium was represented by a monolayer of intestinal epithelial cells derived from 3D intestinal organoids. Additionally, the lamina propria was represented using a PEGDA-GelMA hydrogel, which mimic the subepithelial connective tissue combined with a myofibroblasts monolayer. With this technology, we aimed to provide a culture system with an adequate surface with similar physical characteristics to *in vivo* extracellular matrix (ECM) where, culturing myofibroblasts and epithelial cells in order to study *in vitro* the interaction between these two cell types under physiological conditions. The described experimental setup is represented in *Figure 4*.

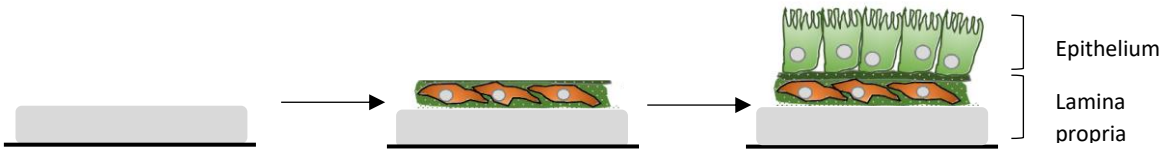

Figure 4. Experimental setup diagram*. The lamina propria is represented by a PEGDA-GelMA hydrogel and a myofibroblasts monolayer. The intestinal epithelium is formed by intestinal epithelial cells derived from intestinal organoid culture.*

In order to achieve our ultimate goal we used the before mentioned *in vitro* mucosa model to perform epithelial migration studies. To study this process, the set up was a little modified. It was used a PDMS stencil for avoid cell growing in the middle of the hydrogel (*Figure 5 and Figure 6*). Performing this change and afterwards using life microscopy techniques, we were able to study the myofibroblasts effects on the epithelium growing and migration.

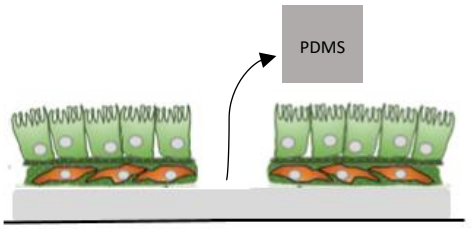

Figure 5. Intestinal mucosa in vitro model modified for epithelial migration studies. *Cells do not grow in the middle of the culture because of a PDMS stencil. When the PDMS stencil is removed, it can be studied how the cells fill this space.*

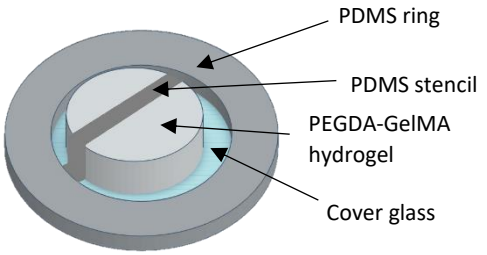

Figure 6. Scheme of the modified experimental setup. *A PDMS stencil is placed across the hydrogel and it is fixed using a PDMS ring.*

2. Hydrogel fabrication

Hydrogels are used for tissue engineering because they offer high similarities to the extracellular matrix such as high amounts of water content and good capacity for nutrient and oxygen transport. Hydrogels can be made of different polymeric materials, which permit to divide them according to their origin, natural or synthetic (El-Sherbiny and Yacoub, 2013). Natural polymers show good bioactivity, biodegradability and biocompatibility but, they have relative poor mechanical properties and immunogenic reactions (Kharkar, Kiick and Kloxin, 2013). On the other hand, synthetic polymers, have better tuneable mechanical and chemical properties and do not produce immune response. Nevertheless, synthetic hydrogels show low biocompatibility and biodegradability (El-Sherbiny and Yacoub, 2013). To generate the hydrogels we used the synthetic polymer Polyethylene-glycol diacrylate (PEGDA) in combination with the natural polymer gelatin methacryalate (GelMA).

Hydrogels were fabricated using photopolymerization (*Figure 7*). This process enables *in situ* formation of cross-linked networks at physiological pH and temperature. It is based on a source of ultraviolet (UV) light applied to the polymer solution, which includes a photoinitiator. Photopolymerization offers several advantages, such as the good spatial and temporal control, low energy requirements, low cost and rapid cure reaction.

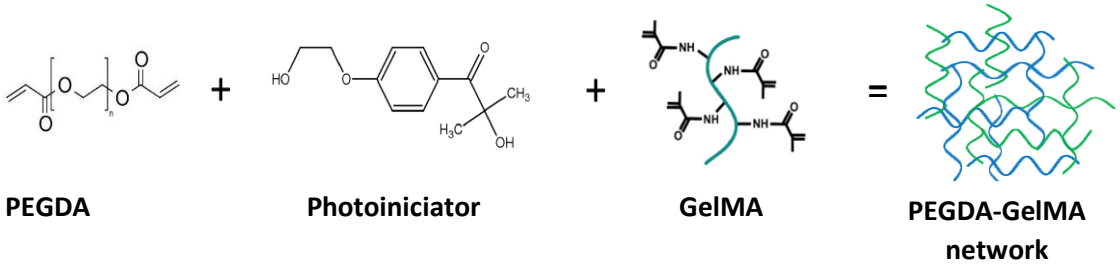

Figure 7. Photopolymerization reaction

We obtain 5%PEGDA-7,5%GelMA hydrogels with 1mm height and 6mm width. Real images of the hydrogels obtained are shown in *Figure 8*.

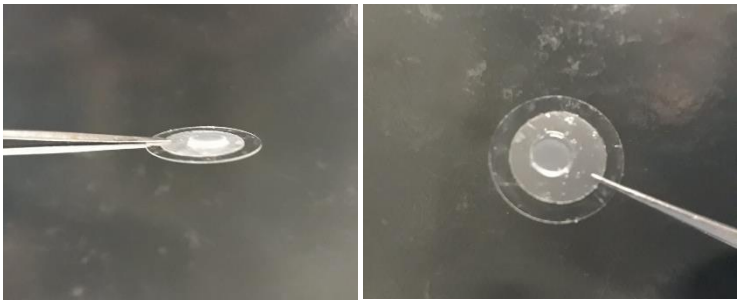

***Figure 8. PEGDA-GelMA hydrogels fabricated in the laboratory**. They are 1mm height and 6mm width*

3. NIH-3T3 fibroblast monolayer characterization

Although the objective of this project was to work with primary myofibroblasts we decided to start setting up the culture conditions to grow fibroblasts on the PEGDA-GelMA hydrogel by using NIH-3T3 cells because they are easier to maintain, have a faster growing ratio and do not have a limited number of passages.

NIH-3T3 fibroblasts cell line was stablished in 1962 from a Swiss albino mouse embryo tissue, and ever since its establishment it has become the standard fibroblast cell line. NIH-3T3 cells divide very fast, with a doubling rate of 20-26 hours. Since we envision to culture epithelial cells on top of the NIH-3T3 layer, the fast proliferation ratio could affect the subsequent formation of the epithelial monolayer. Keeping this in mind, we decided to arrest the cell cycle of the NIH-3T3 cells by treating them with Mitomycin C (MMC) drug. MMC is an alkylating agent that cause the cross-linking of DNA and inhibition of DNA synthesis preventing cell division.

We seeded NIH-3T3 treated and non-treated cells on the PEGDA -GelMA hydrogels as explained in section 7 of materials and methods. The representative bright field microscopy images shown in *Figure 9* demonstrated that in both conditions, after 24 hours of seeding NIH-3T3 cells were perfectly attached to the PEGDA-GelMA hydrogel material and formed a uniform monolayer that covers the whole surface of the hydrogel disk.

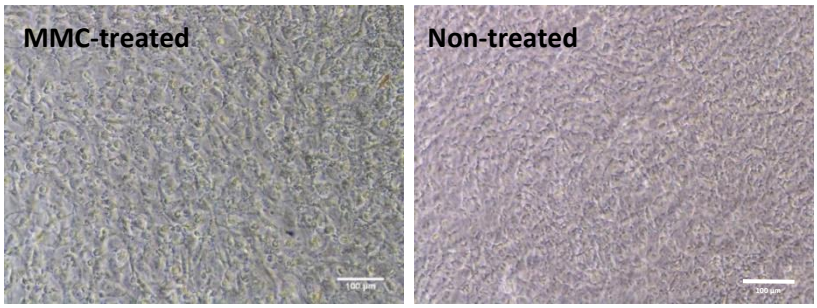

Figure 9. NIH-3T3 fibroblasts monolayer. *Both conditions were studied by microscopy. 24 hours after the seeding both MMC-treated and non-treated fibroblasts were perfectly attached to the hydrogel surface. It could not be seen any difference between the two conditions. Scale bars: 100µm*

Fibroblasts are commonly used as feeder layers, because they are specialized in secreting extracellular matrix (ECM) proteins providing a supporting ECM for other cell types to grow. In or studies, fibroblasts allow the growing of intestinal epithelial cells. For this reason, before going a step further and seeding epithelial cells on top of these samples, we wanted to ensure that the cultured fibroblasts were actively expressing ECM proteins. We analysed by immunofluorescence the expression of fibronectin and type IV collagen, which are two important proteins of the ECM. Representative immunofluorescence images in *Figure 10* clearly show that MMC-treated cells expressed more fibronectin and type IV collagen than the non-treated fibroblasts. In order to characterize these differences, it was done an ECM quantification where the amount of fibronectin and collagen was analysed. In *Figure 11* is represented the accumulated amount of each protein at days 1 and 3 of culture. Our results clearly show that at day 3 of culture MMC-treated NIH-3T3 cells doubled the amount of collagen and fibronectin expression in comparison with non-treated NIH-3T3 cells (*Figure 11b*). These results indicate that when NIH-3T3 cells cannot divide might spend more energy in doing other cellular activities such as secreting ECM proteins. We were interested to see the expression levels of ECM proteins also at day 1 of culture. We reasoned that it would be interesting to know the shorter time the fibroblasts needed to secrete extracellular matrix in our system, in order to determine the earliest moment to seed the epithelial cells on top. On the one hand, our results show that after 1 day of culture, MMC-treated cell appeared to be healthier than at day 3 of culture, suggesting that seeding the epithelial cells on top of the fibroblasts after 1 day of culture could favour the viability of the co-culture. Regarding protein secretion, we found that at day 1 of culture, the amount of fibronectin expression was much less than at day 3 (*Figure 11a*). These results

indicate the amount of ECM protein secretion is proportional to the culture time. We also observed that after 1 day of culture MMC-treated fibroblasts were expressing four times more fibronectin than non-treated fibroblasts (*Figure 11b*). From these results we can hypothesize that while non-treated cells divided once or even twice in 24 hours MMC-treated cells keep on producing matrix without spending time on cellular division. Since the difference in ECM protein expression between treated and non-treated NIH-3T3 cells is more pronounced at day 1 than at day 3 of culture (*Figure 11b*) we believe that the advantage that the division blockade gives to the ECM protein secretion is masked by the increased of cell density in non-treated cells samples with time.

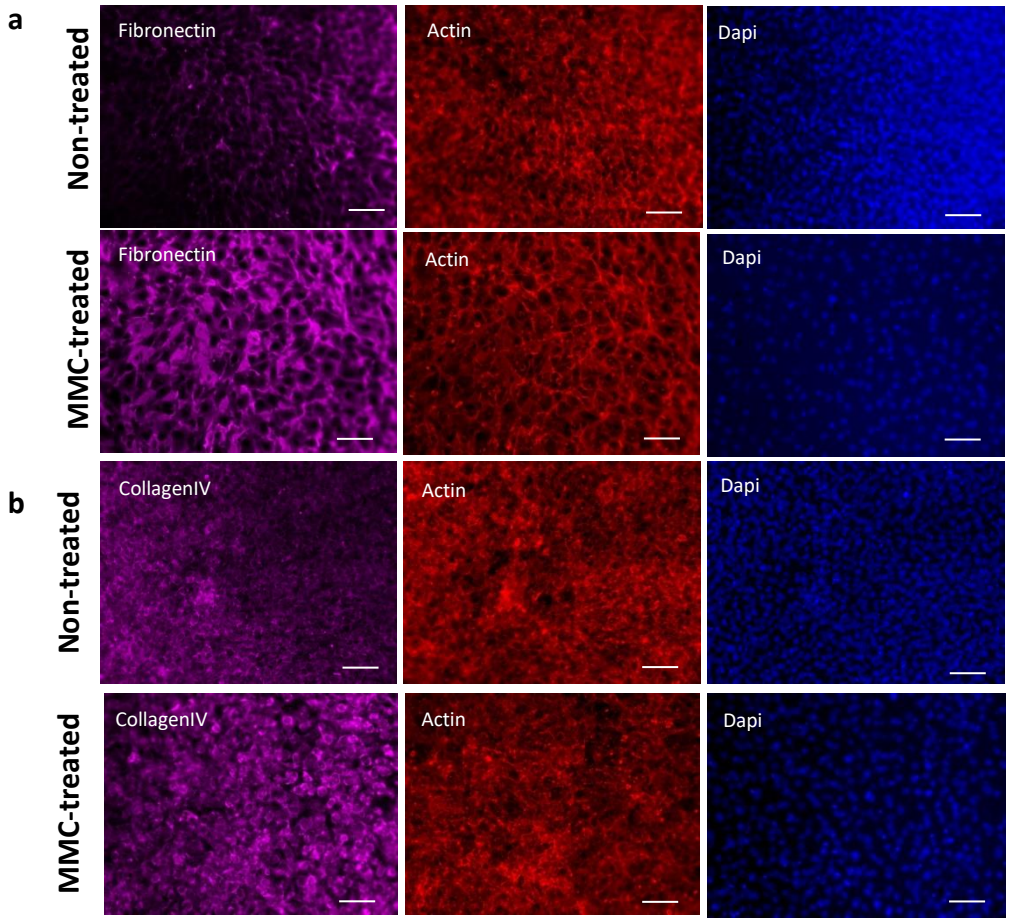

Figure 10. NIH-3T3 fibroblasts monolayer characterization staining at 3 days in culture. *a) fibronectin (magenta), actin (red) and DAPI (blue) staining. b) type IV collagen (magenta), actin (red) and DAPI (blue) staining. Scale bars: 100µm*

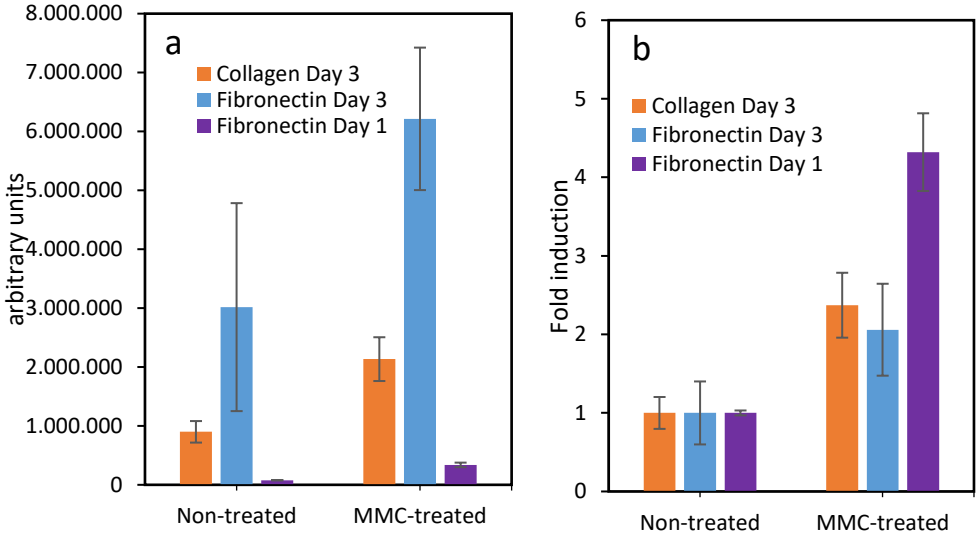

***Figure 11. ECM quantification**. a) Accumulated amount of fibronectin and type IV collagen. b) Fold induction. (In all the different conditions studied, p<0,05).*

4. NIH-3T3 fibroblasts and epithelial cells co-culture characterization

Epithelial cells were seeded on NIH-3T3 fibroblasts covered hydrogels

Once the NIH-3T3 fibroblast monolayer was characterized, epithelial cells were cultured on top. These epithelial cells were obtained from intestinal organoids digestion according to the protocol described in section 4 of material and methods.

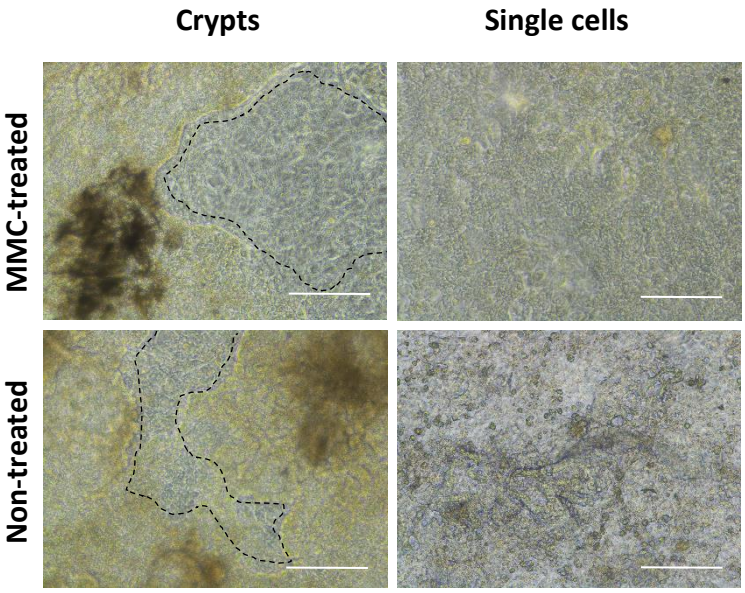

***Figure 12. Epithelial monolayer formation at day 5 of culture.** Into the dotted lines it is shown the new epithelium formation. In single cells samples the epithelial monolayer cannot be distinguished because all the space is fully covered. Scale bars: 100µm*

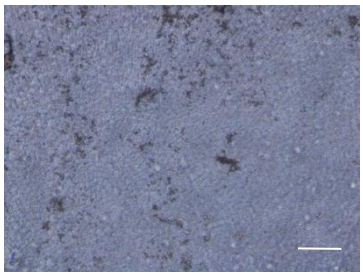

***Figure 13. Control hydrogel without feeder layer**. Intestinal single cells do not attached (Day 1 of culture). Scale bars: 100µm*

We analysed two conditions, seeding single cells and seeding crypts. The objective was to determine the best protocol for obtaining a uniform epithelial monolayer (*Figure 12)*. In both conditions, the single cells and crypts attached to the NIH-3T3 feeder layer and proliferate forming an epithelial monolayer. Notice that intestinal epithelial cells could not attach and grow on a hydrogel without a feeder layer (*Figure 13*). When no feeder layer was present, no cells attached to the hydrogel and most of them died after 1 day in culture. This control demonstrates that epithelial cells cannot attach directly to the hydrogel making evident the need of fibroblast feeder layer for the epithelial layer formation on the PEGDA-GelMA hydrogel.

Epithelium coverage quantification in NIH-3T3 fibroblasts and epithelial cells co-cultures

Finally, we quantify the epithelial coverage of each hydrogel with the purpose of determining which of the tested condition was giving the best results regarding epithelial monolayer extension and homogeneity. Since monitoring and characterizing the epithelial monolayer formation under the bright field microscopy was challenging due to the difficulty to distinguish the epithelial cells on top of the NIH-3T3 fibroblasts, we thought to perform specific epithelial immunofluorescent stainings of our co-cultures.

β-catenin is an integral structural component of cadherin-based adherents junctions and therefore an specific marker of epithelial cells (Valenta, Hausmann and Basler, 2012). *Figure 14* shows a reconstruction of different stitched pictures that allow the visualization of the complete hydrogel (6mm of diameter) for each of the analysed conditions. We quantify the percentage of disk coverture by epithelial monolayer using the β-catenin staining. Notice that the NIH-3T3 fibroblast cells were not positive for this marker. Our results show that the best coverage was obtained in the samples with intestinal single cells seeded. The samples with non-treated feeder layer with single cells seeded on top had 87% of coverage. Followed by MMC-treated feeder layer with single cells seeded samples with a 70% of coverage. The non-treated feeder layer with crypts seeded samples obtained a 55% of coverage while the MMC-treated feeder layer with crypts seeded had a 51%.

We can conclude that to seed intestinal single cells instead of crypts is definitely the best condition for obtaining a more homogenous epithelial layer and a more covered hydrogel. For explaining this clear difference, it can be suggested that when single cells were seeded, the stem cells were very distributed on the hydrogel and it was translated into a homogeneous growing and expansion of the epithelium. In contrast, when crypts were seeded, the proliferative cells were compacted in these crypt-like structures resulting in an irregular distribution of the stem cells. Subsequently, it caused the asymmetrical epithelium growing on these samples. Moreover, MMC-treatment of the NIH-3T3 cell does not affect the epithelial monolayer formation. Even there is a clear effect of MMC treatment on ECM proteins expression in NIH-3T3 fibroblasts, probably this effect get diluted with time because of the growing of non-treated fibroblasts. We can assume that as much cells grow more ECM was being accumulated on the hydrogels meaning that at some time point, the difference between the amount of ECM proteins in both conditions was despreciable.

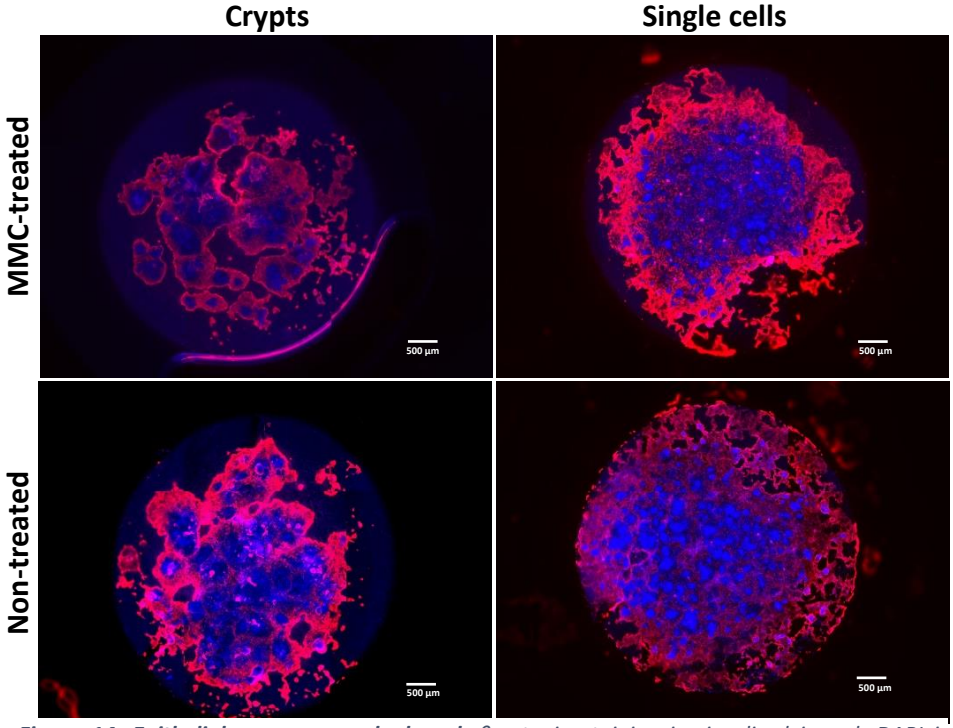

Figure 14. Epithelial coverage on hydrogel. *β-catenin staining is visualized in red. DAPI is visualized in blue. It can be observed the entery hydrogel surface and the epithelium extension. Scale bars: 500µm (day 7 after seeding epithelial cells)*

Overall, form our experiments employing NIH-3T3 as feeder layer we concluded that the best epithelial seeding condition is the one using epithelial single cells. Thus, we will continue our research employing only this technique.

5. ISEMFs culture characterization

ISEMFs characterization

As we mentioned before, our ultimate goal was to generate an *in vitro* intestinal epithelial mucosa model to study the potential contribution of the intestinal epithelial myofibroblasts to the expansion and migration of the intestinal epithelial cells. Myofibroblasts cells are more physiologically relevant than a fibroblasts cell line. With using them, we can better mimic the intestinal mucosa behaviour.

After isolate ISEMFs, it was required to characterize these cells, in order to be sure that they, in fact were myofibroblasts. For these reason an immunostaining was made with α-SMA, vimentin and desmin antibodies.

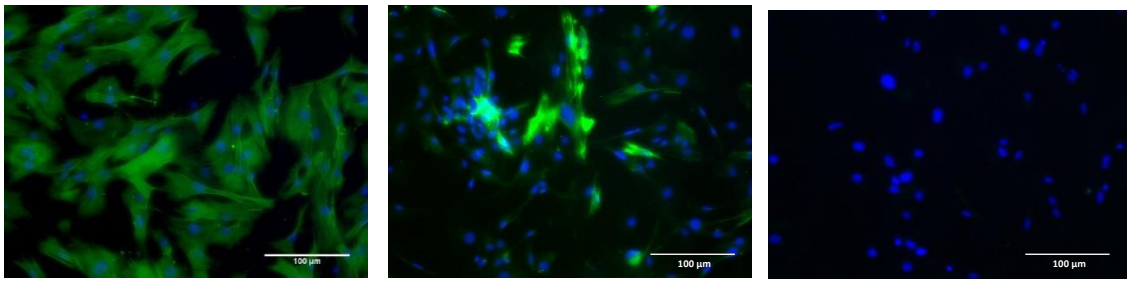

***Figure 15. Myofibroblasts characterization**. a) α-SMA staining; Myofibroblasts are positive to these smooth muscle cells marker. b) Vimentin; they are also vimentin positive (vimentin is a fibroblasts marker). c) Desmin staining; myofibroblasts are negative to this smooth muscle cells marker. By these stainings we can conclude that these cells are actually myofibroblasts. (cell nucleus were stained with DAPI (blue)). Scale bars: 100µm*

The result is shown in *Figure 15.* These cells were α-SMA+, vimentin+ and desmin-, ensuring that they were myofibroblasts.

Notice that ISEMFs were not treated with MMC. In this case, it was not necessary to inhibit cell division because ISEMF are primary cells that grow very slowly and have a limited number of passages. Thus, there were no reason to use this drug.

Subsequently, the ISEMFs were cultured on PEGDA-GelMA hydrogels and, as we previously observed in NIH-3T3 cultures, these cells perfectly attached and grew until covering the entire hydrogel surface (*Figure 16*).

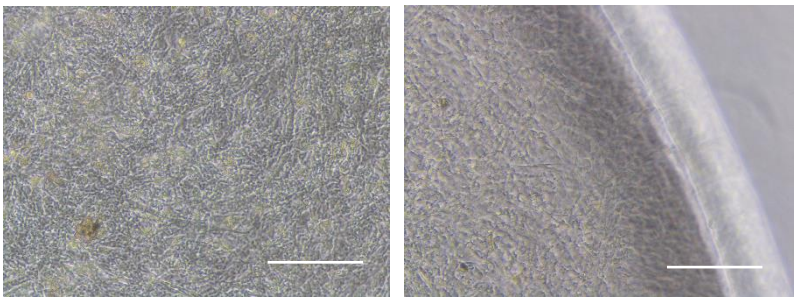

Figure 16. Myofibroblast monolayer covering the PEGDA-GelMA hydrogel surface. *The images show ISEMF culture on hydrogels at day 3 of culture. The cells look very healthy and homogeneously distributed. Scale bars: 100µm*

6. ISEMFs and epithelial cells co-culture characterization

Once we had the monolayer of ISEMFs feeder layer on the PEGDA-GelMA hydrogel formed, we seeded intestinal single cells derived from intestinal organoids as described in section 4 of the Material and Methods. After seeding single cells on myofibroblasts monolayers, we observe the formation of small cyst-like structures (*Figure 17a*). This result agree with some previously results observed on the literature (Lahar *et al.*, 2011). As is explained in the introduction, it is known that ISEMF secret some factors that favour stem cells growth, for instance Wnt2b or Wnt4. For these reason, we hypothesize that these cyst-like structures are formed by proliferative cells. Interestingly, this effect was not observed in previous studies with NIH-3T3 fibroblasts and epithelial cells, pointing out that this behaviour is specific of ISEMF and not from all types of fibroblasts.

In addition, it is important to emphasise that the effects of ISEMF on the epithelium growing and migration is depending on the ISEMF isolation used. As is previously mentioned, primary myofibroblasts have a low division ratio and a limited number of passages. These characteristics restrict the number of experiments that can be done with the same isolated cells, meaning that we are subjected to the intrinsically variability of each isolation.

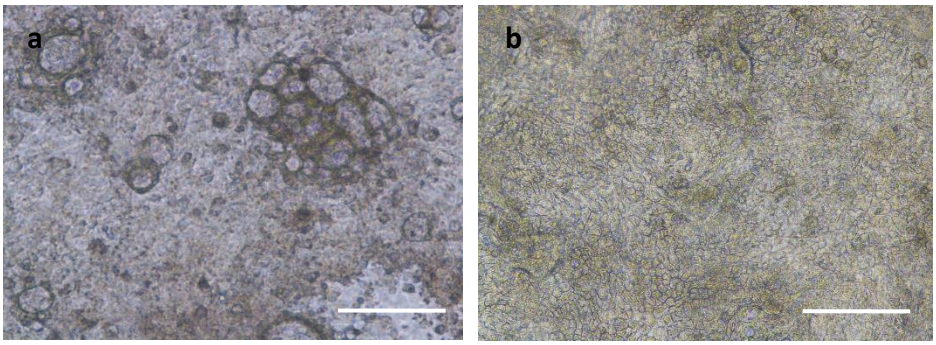

Figure 17. Myofibroblasts and epithelial cells co-culture at day 1. *a) It was observed small cyst-like formations at day 1 of culture. b) At day 5 of epithelial cells culture, this cysts have disappeared and in it's place have been formed a really nice epithelium layer. Scale bars: 100µm*

Epithelium coverage quantification on ISEMFs and epithelial cells co-cultures

After performing these co-cultures we analyse the coverage. In the same way as we did in NIH-3T3 experiments, we stained the specific epithelial marker β-catenin in order to study the epithelial monolayer over the ISEMFs.

The results show that the 74% of hydrogel surface was covered by the epithelial monolayer. Moreover, this epithelium was very homogenous (*Figure 18*). This result evidence again that seeding intestinal single cells is a good technique for obtaining a high coverage percentage at day 7 after epithelial cells seeding.

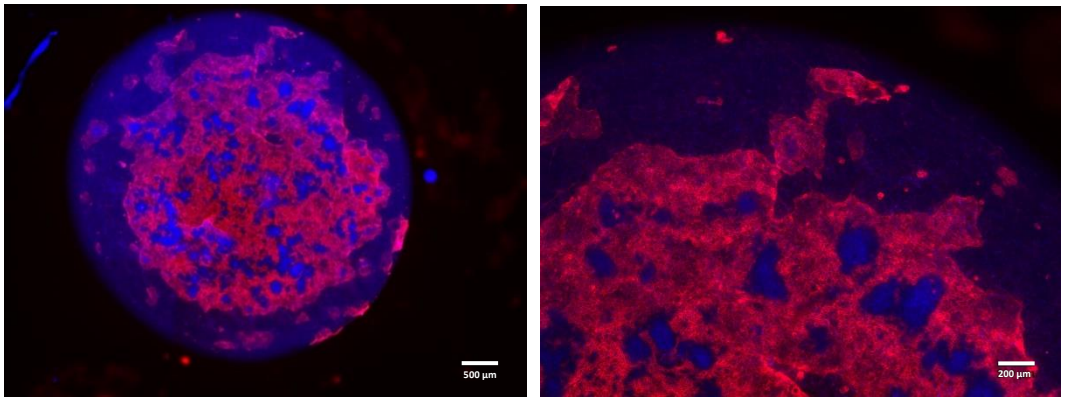

Figure 18. Epithelial coverage on hydrogel*. β-catenin staining is visualized in red. DAPI is visualized in blue. In the left image is shown the entire hydrogel, it is clearly observed that most of the surface is covered by the epithelium layer (scale bar: 500µm). The right image shows the quality and shape of the epithelium (Scale bar: 200µm) (these cultures were stopped at day 7 after epithelial cells seeding)*

Analysis of ISEMFs behaviour when they are in contact with epithelial cells

In order to analyse the relationship between the ISEMF and the epithelial cells we performed a series of immunostainings employing specific-cell markers. Specifically, we used β-catenin and α-smooth muscle actin (α-SMA) antibodies to stain the co-culture. β-catenin is an integral structural component of cadherin-based adherents junctions and therefore a marker of epithelial cells (Valenta, Hausmann and Basler, 2012). On the other hand, α-SMA is the actin isoform that predominates within vascular smooth-muscle cells and myofibroblasts. Moreover, the expression of α-SMA correlates with the activation of myofibroblasts (S. Cherng et al., 2013). The results are shown in *Figure 19*. As is observed in *Figure 19a,* ISEMFs, which are labelled with α-SMA staining, were covering the entry hydrogel surface. The epithelium, represented in Figure 19b using β-catenin staining, has been formed over the ISEMFs. Interestingly, we observed that the

ISEMFs that are in tight contact with the front of migration of the epithelium are placed longitudinally surrounding all the epithelial cells while are more spread and expressing more α-SMA. Nevertheless, we observed that ISEMFs placed just beside the epithelial front of migration were perpendicularly aligned with the epithelium, clearly expressing stress fibres and really spread. This observation suggest that intestinal myofibroblasts might actively pull the intestinal epithelial cells that form the monolayer contributing to its migration by exerting mechanical forces.

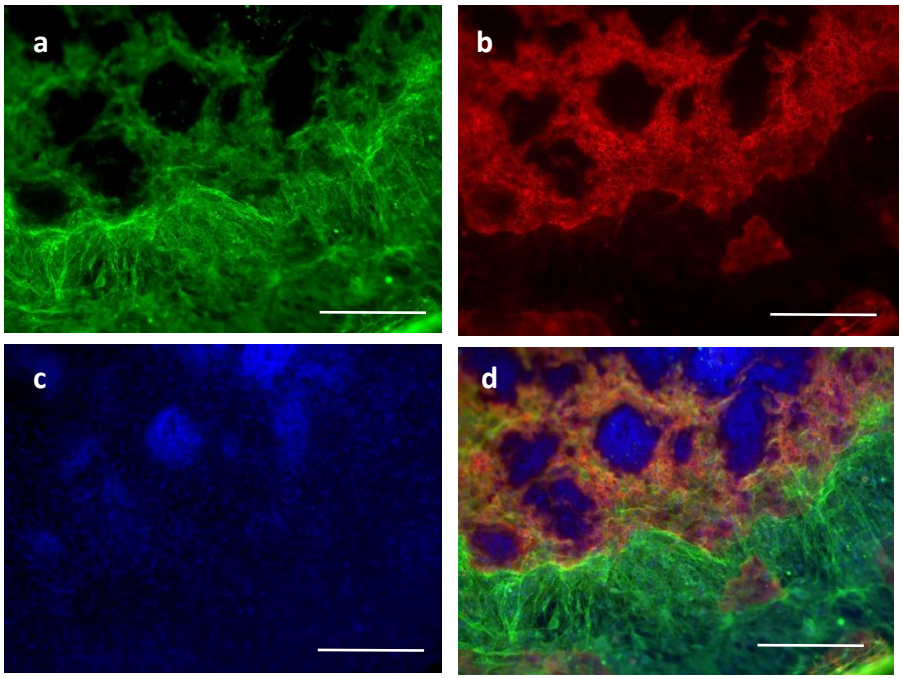

Figure 19. Co-culture characterization staining. a) *Myofibroblasts (green) are clearly organized around the epithelium (red).b) β-catenin staining shows the epithelium formed. c)cell nucleus were stained with DAPI (blue) and is clearly shows that throughout the culture we found loci of compacted cells, which were not positive either for SMA or for B-catenin. d) merged image (Scale bars: 100μm)*

As is clearly represent in *Figure 19c*, in all the different samples studied there were many accumulations of cells clearly stained with DAPI. But, they were not stained with any antibody used (*Figure 19d*). Even we did not know if these aggregates were fibroblasts or epithelial cells, it can be suggested that, where a stem cell attached, proliferate and form this cell aggregates while the descendent cells start differentiating and forming the epithelial monolayer.

Some similar results were seen on literature. Thorne et al. cultured monolayers of intestinal epithelial cells that come from organoids (Thorne et al., 2018). In their cultures, they could obtain epithelial monolayers with distinct dense compartments identically as the cultures that we obtained. Just as we previously supposed, they report

that these cell clusters are aggregates of stem cells and Paneth cells. Regarding these information, we hypothesize that the cell clusters present in our cultures are crypt-like zones with proliferative and Paneth cells. In this way, we can assume that the absence of β-catenin staining is because proliferative cells do not express as much cell junction proteins as differentiated enterocytes.

7. Epithelium migration analysis

ISEMFs labelling with PKH26 red fluorescent cell linker

Our previous experiments suggested that myofibroblasts might contribute to the epithelial monolayer expansion and migration by exerting mechanical forces. In order to further investigate this phenomenon, we slightly modified our model. We employed the co-culture model that we had stablished, but for these experiments we used a PDMS stencil that we placed across the hydrogel, as indicated in the scheme of *Figure 6* (previously explained in section 1 of results and discussion). The stencil physically impeded the attachment and growth of cells on a specific area of the hydrogel. After cells attached to the hydrogel, we removed the stencil and analysed the expansion and migration of the epithelial on top of the fibroblasts. In order to better distinguish the two cell types in our life microscopy experiments was decided to label the ISEMFs membrane with the cell membrane dye PKH26. PKH26 is a type of lipophilic dye with long aliphatic tails that incorporates into lipidic regions of the cell membrane and emit red fluorescence. It can conduct fluorescence labelling for numerous type of cells. The optimal concentration of PKH26 dye could change between different cell types because it is depending on cell number, cell dimensions and the final volume of the reaction. After trying different conditions, we determined that the best conditions for a proper ISEMF membrane staining were a cell concentration of 1.250.000 cell/mL, a total volume of 200µL and a dye concentration of 10µM (*Figure 20*).

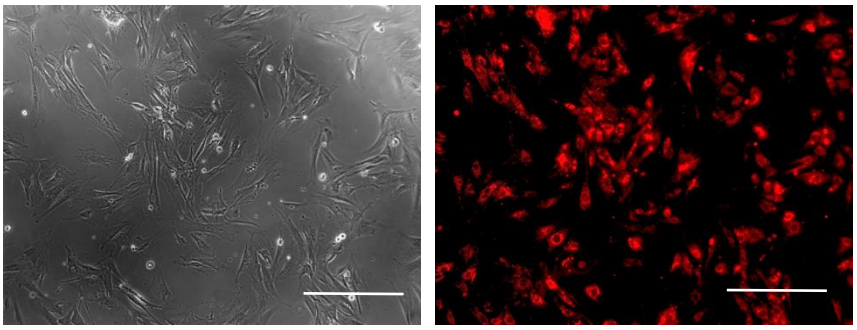

Figure 20. PKH26 staining. *The left image shows a bright field view of the stained ISEMF. The right image shows the quality of the PKH26 staining. Scale bars: 100µm*

The ISEMFs role in epithelium migration

First, it has to be pointed out that this experimental setup was technically challenging. For this reason, the following reported experiment reflect just the preliminary results obtained after the first trial. Even though the experimental setup did not work 100% the way we originally planned, we could make some interesting observations. *Figure 21* shows a summary of the observations about the epithelium migration during a 15 hours live imaging video. At time 0, we observed that ISEMF were accumulated underneath of the epithelium and during the first 4 hours they spread out polarized in epithelium migration direction connecting to epithelium pieces, just as we observed in co-culture characterization. Then, the epithelium layer start migrating only in the direction where the ISEMF were moving. With the pass of the time it can be clearer observed that myofibroblasts (some of them marked with dark arrows on *Figure 21*) were really spread and tensioned while connecting two pieces of epithelium, clearly suggesting that they are actively pulling trying to join them.

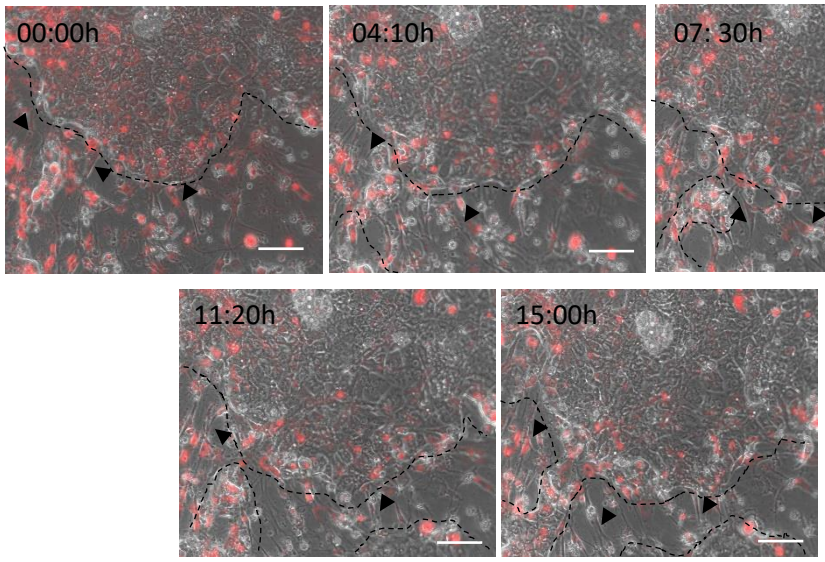

Figure 21. Epithelium migration*. It can be seen at different time points how the empty space is fill at first by myofibroblasts and after by the epithelium that is being pulled by the myofibroblasts. Scale bars: 100µm*

For ensuring that in fact ISEMF migrate, we analysed these experiment results just with fluorescence imaging. In *Figure 22* are shown the ISEMF stained with PKH26 dye at time 0 and 15 hours. It could be observed that at time 0 most of the cells were accumulated on the top left area of the image whereas after 15 hours the cells were more distributed through all the image area. This result strongly suggest that ISEMF actively migrate before or at the same time as epithelium does.

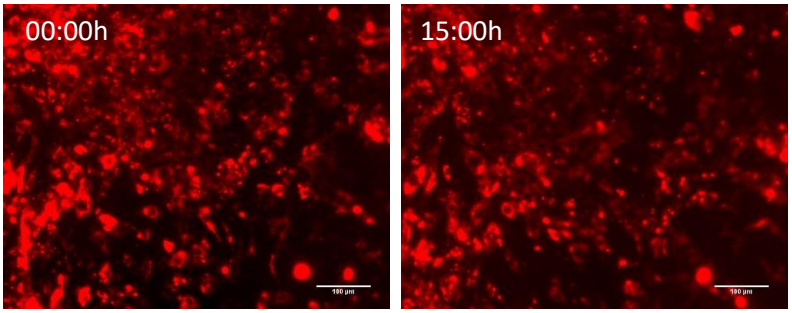

Figure 22. ***ISEMF stained with PKH26 dye during migration experiments.*** *At time 0 most of ISEMF were accumulated on the top left area of the image while after 15 hours the cells were more distributed meaning that they actively migrate underneath the epithelium. Scale bars: 100µm*

Conclusions

The main conclusion of this project is that we have generated an intestinal mucosa *in vitro* model using different components: in one hand, we have fabricated a PEGDA-GelMA hydrogel that we have used to mimic the ECM and also we have been able to grow an ISEMF layer. Using these two components, we have mimicked the intestinal lamina propria. On the other hand, we have represented the intestinal epithelium culturing an intestinal epithelium layer over the ISEMFs. This new stablished model, can be useful for different physiological or pathological studies. In this context, we have stablished the conditions to adapt this model for migration studies.

The conclusions we obtained from the experiments we performed are the following:

- MMC treatment enhance the expression of fibronectin and type IV collagen in NIH-3T3 cells, but this treatment do not affect the epithelial cells attachment, proliferation or migration.
- Intestinal epithelial cells only can grow on the hydrogels using a fibroblast feeder layer, denoting that they have an important supporting function.
- The immunostaining results using specific epithelial and fibroblasts markers show that ISEMF that are in contact with the epithelium are more activated and have a particular disposition.
- The live imaging results validate the previous immunostaining results and reinforce the idea that ISEMFs actively pull the epithelial monolayer guiding the migration.

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Self-assessment

This project has been performed during my internship in Biomimetic Systems for Cell Engineering group that belongs to the Institute for Bioengineering of Catalonia (IBEC). This opportunity has allowed me to know the world of basic research into a much deeper level and it has also been a very important step in my way to the professional world. In the same way, I have been able to understand the amount of people and years of work that each article or scientific discovery represents. That, allow me to understand the scientific literature from a more professional point of view.

This internship has allowed me to get the capability to reason much more about the results obtained with the different experiments I performed. Additionally, performing this work, I have learned a lot in the area of tissue engineering and, at the same time, it has helped me to refresh and understand clearer concepts and laboratory techniques learned during my university studies.

At the same time, having achieved most of the planned objectives in a satisfactory way has been a personal enrichment, especially because the work I performed during these months can be useful for further investigations in this research group.

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