



Universidad de Valladolid

**TRABAJO DE FIN DE MASTER**

**Master in Biomedical Research**

# Study of Human Epithelial Corneal Cells Regeneration on Silk-Fibroin-PEG Membranes.

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**Supported by European Research Council 2018-ADGSILKEYE-833106.**

# Abstract

The aim of the present work is to analyze the regeneration capabilities, as well as metabolic behavior and cell characterization of corneal epithelial cells on a Silk fibroin with 5% PEG membrane, before and after suffering an injury. The cells were analyzed and tested for up to 48 hours to properly assess wound regeneration. Microphotographs of these *in vitro* samples were realized every 24 hours and immediately after wound induction, cells reached confluency on the biomaterial substrate, as well as a physiological substrate. For cell characterization, we used the following immunohistochemistry: Cytokeratin 12 (CK12), Focal adhesion Kinase (FAK), αSMA, Phalloidin, and DAPPI. For metabolic essays, the WTS-1 test was used. To properly see the morphology Scanning and Transmission, Electronic Microscope images were acquired and analyzed. The Silk Membrane with 5% PEG was able to be as efficient as a simulated physiological ambient, as far as wound healing capacity goes, and showed promising signs of its use as a biomaterial having the same time to close wounds as other collagen-based materials. When the metabolic activity was regarded, it was possible to see a similar rise on both substrates showing again a good indicator for its clinical use. Nevertheless, future studies are needed to correctly determine the viability of this biomaterial *in vivo* models, for its consequences and methods of manipulation may widely differ for possible clinical use. However, it is possible to conclude that the Silk membrane with 5% PEG has great potential for future use in corneal damage.



**Keywords:**

Corneal epithelial cell, silk fibroin, membrane, PEG, corneal healing, cell proliferation, cell migration.

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| **HEC**: | Human Epithelial Cells |
| **ECM**: | Extracellular matrix |
| **CK12**: | Cytokeratin 12 |
| **FAK**: | Focal Adhesion Kinase |
| **CK3**: | Cytokeratin 3 |
| **GFs**: | Growth Factors |
| **SFM**: | Serum-Free Medium |
| **PBS**: | Phosphate Buffered Saline |
| **TGF**: | Transforming Growth Factor |
| **PDGF**: | Platelet-Derived Growth Factor |
| **KGF**: | Keratinocyte Growth Factor |
| **PEG**: | Polyethylene Glycol |
| **HB-EGF**: | Heparin-Binding EGF-Like Growth Factor |
| **FGF**: | Fibroblast Growth Factor |
| **IGF**: | Insulin-Like Growth Factor |
| **DAPPI**: | 4`6 – Diamidino – 2 Phenylindole |
| **DMSO**: | Dimethyl sulfoxide |
| **DMEM/F-12** | Dulbecco´s Modified Eagle Medium: Nutrient Mixture F12 |
| **WTS - 1** | Colorimetric assay based on the conversion of tetrazolium salt into a colored dye. |
| **SEM** | Scanning Electronic Microscope |
| **TEM** | Transmission Electronic Microscope |
| **SF-PEG** | Silk fibroin membrane with 5% PEG |

# 1. Introduction

## 1. Corneal structure

The human eye is a specialized sensory organ, capable of receiving light stimuli, which will later be converted into visual images in the brain. This process composes 80% of our global world perception, and its primary action mechanism is the optical refractive capacity, in which radiance can properly be focused on the retina.

The outset and anterior part of the eye is the cornea. It has the dual function of protection when regarding the inner contents of the organ, as well as providing for almost two-thirds of the eye refractive capability (Eghrari et al.,2015). The human cornea is composed of five layers: Epithelium, epithelial basement, membrane, Bowman´s layer, Stroma, Descemet membrane, and endothelium (Bueno et al.,2011).

The corneal epithelium is composed of four layers of non-keratinized, stratified squamous epithelial cells, measuring approximately 50 µm in thickness.It is composed by three layers of squamous cells. Connected by zonula ocludens and provided with microvilli. The middle two to three cell layers create the wing layer, and the innermost cell layer is the basal layer or germinative layer where mitosis occurs.. The next layers are that adhere to the underlying basement membrane by adherent junctions. The anchoring complex further down will be responsible for linking the cytoskeleton of these basal epithelial cells and the stroma, it's made of hemidesmosomes, anchoring fibril, and another complex named anchoring filament complex (Masters, 2009).

The stroma is the corneal structural framework, it measures 500 µm, representing 90% of the corneal volume, and is composed of a heterodimeric complex of Type I-V collagen and plays a pivotal role within the eye, for it has to combine precise shape with an almost full transmission of visible light, because it is now known that these arrangements in lamellae provide not only the shape, but the mechanical properties of this tissue, injunction with water and keratan sulfate proteoglycans; such as Lumican, keratocan, and mimecan; and proteoglycans such as Decorin, however, the concentration of proteoglycans varies. The stroma has a crucial part to play in the corneal histology, for it helps to give mechanical strength to the cornea, it's the major tissue when regarding transparency, as well as acts as the main refracting lens (Bron, 2001)( Meek, K. M.; Carlo K., 2015).

The following layer is the Descemet membrane, a basement membrane of endothelial origin, both are produced by the endothelial cells of the cornea. Having a thickness of approximately 8 µm and a composition of mostly collagen, as well as other extracellular matrix components. It acts as a barrier against the passage of substances between the corneal stroma and the aqueous humor located in the anterior chamber of the eye.

Finally, the innermost layer of the cornea is the endothelium, composed of a single layer of cells that rests on the Descemet membrane. The endothelium is responsible for the maintenance of the clarity of the cornea by regulating the water content of the stroma. It achieves this through its pump and barrier functions, which work together to transport water from the stroma to the aqueous humor. The endothelial cells are tightly packed together and are connected by tight junctions, preventing the movement of substances between them and humidity control (Waring et al., 1982).

## 2. Healing process

Because the cornea is an extremely specialized structure, that plays a primary role in image acquisition, the integrity of its tissues is essential to its proper functionality, meaning that any injury or damage can lead to serious vision impairment. Indeed, the wound healing process is key in the

corneal structure regeneration process for it involves a series of complex events that enable the correct replacement of the damaged cells with healthy ones. Epithelial cells, as discussed before, are an essential component of the cornea, and as such, have a crucial role in the wound-healing process (Kuwabara et al., 1976).

Epithelial cell migration is a critical step in the corneal wound healing process, involving a complex interplay between the extracellular matrix, cell signaling pathways, and cytoskeletal rearrangements. In this process of corneal healing, epithelial cells undergo significant changes in their migratory pattern, leading to a formation of migratory fronts that tend to move toward the center of the injury and involve the activation of integrins, which are cell surface receptors that interact with the extracellular matrix to facilitate cell adhesion and migration (Stepp et al., 2014).

Cell proliferation is another crucial aspect that plays a major role in the healing of cornea, while loss of cells due to injury cannot be replaced by cell division alone, recent studies show that the healing process does involve a major proliferation characteristic, usually being a process that involves the activation of different signaling pathways that promote the proliferation and differentiation of these cells and are maintained by a variety of extrinsic and intrinsic factors, such as the local environment, survival factors, and cytokines. Although several markers have been used to quantify this process, because of several other growth factors, matrix, degradation proteins, and receptors being involved in this healing, it still remains a very complex and not so defined process (Daniels et al., 2001).

Because of this, understanding the mechanisms that drive and modulate corneal wound healing is essential for the development of state-of-the-art therapies to treat corneal injuries and diseases (Hertsenberg et al., 2017).

## 3. Interaction between the extracellular matrix and cellular characterization

Epithelial cells are characterized by their presence of a large nucleus and polar arrangement, having a defined structural orientation with both sides of the membrane possessing distinct numbers and types of proteins. The corneal epithelial layers are constantly renewing themselves, this process can be basal, for the natural removal of cells, as well as in the wound healing process, in both situations, the stimulation of growth factors and environmental stress that switch the basal response to stimulatory signals and expression of genetic based responses (Shanli et al., 2016).

The extracellular matrix (ECM) comprehends a complex network of molecules that are deeply responsible for tissue development, maintenance, and repair, as well as cellular pathway signaling. The ECM is particularly essential in the process of differentiation, wound healing, and function in epithelial cells, especially when regarding the cornea tissue (Tanihara et al., 1997). Furthermore, the ECM plays a major role in corneal tissue development, in which intracellular unions and communication are key components, making clear that further study and analysis of this variable should be considered when studying a healing scenario.

Cytokeratin, a group of intermediate filament proteins found in the epithelial cells, are markers that permit the correct analysis of cellular behavior in wound healing, especially Cytokeratin 3, a marker of corneal epithelial cell differentiation and Cytokeratin 12, which is specific to the cornea, allowing the identification of cells stress-induced response and its consequences on the regeneration process (Daniels et al., 2001).

Focal adhesion kinase (FAK) is a signaling molecule that has been associated with critical roles in the formation and maintenance of membrane junctions, and by measuring the phosphorylation state of FAK, it’s possible to classify the strength of said junctions and their role in maintaining the integrity

of the epithelial layer (Teranishi et al., 2009). To better see and analyze the arrangement and organization of microfilaments, an electronic microscope is commonly used to see the structural integrity of the epithelial layer and ensure proper cell function and characterization.

### 3.1. Growth factors

There is enough accumulated evidence to show that GFs promote the proliferation and induce the migration of corneal cells, as well as the maintenance of corneal transparency, when in a injury scenario (Imanishi et al., 2000). Such GF can be differentiated in those secreted by the epithelium, such as Transforming growth factor-β1 and Transforming growth factor-β2 (TGF- β1; TGF-β 2) and Platelet-derived growth factor (PDGF), and those produced by fibroblasts such as keratinocyte growth factor (KGF), hepatocyte growth factor (HGF), and epidermal growth factor (EPG).

Growth factors (GF) are also highly considered to play a key role in corneal healing The EGF family is usually linked with the ability to increase corneal epithelium proliferation and migration, as well as an inhibitor of the differentiation-related marker keratin K3. This family is composed of 13 members, but the main ones involved in epithelial wound healing are EGF, transforming growth factor-α (TGF-α), and Heparin-binding EGF-like growth factor (HB-EGF) (Mukerji et al., 2003).

EGF is secreted in fibroblasts, macrophages, and platelets and seems to influence epithelial cells in a paracrine fashion, while by itself cannot stimulate epithelial wound closure, if delivered in a controlled released fashion can accelerate the wound healing process. On the other hand, PDGF, TGF- α and HB-EGF can directly induce cell proliferation and migration, furthermore, HB-EGF signals adjacent cells in a juxtracrine manner, having high mitogen and chemo-attraction capabilities for epithelial cells, making all of them essential for epithelial wound healing (Yu et al., 2010).

There is enough accumulated evidence to show that GFs such as epidermal growth factor (EGF), fibroblast growth factor (FGF), transforming growth factor (TGF), Keratinocyte growth factor (KGF), hepatocyte growth factor (HGF), platelet-derive growth factor (PDGF) and insulin-like growth factor (IGF) promote the proliferation of corneal cells and induce the migration of corneal cells, as well as maintenance of corneal transparency (Imanishi et al., 2000).

## 4. Fibroin silk membrane as a biomaterial

One topic that has gained significant attention in regenerative medicine in recent years is the use of biomaterials in the medical and research area, specifically, scaffold materials that aim to promote tissue regeneration. The scaffold material will influence the response of cell types cultured within, and the host response in patients, and when regarding its use on the cornea, the materials applied should be, or become, transparent when implanted, as well as present enough mechanical strength to endure suturing or gluing processes (Palchesko, et al., 2018). The most commonly used materials when regarding corneal application are amniotic membranes, decellularized corneas, collagen materials, and silk films, for the unique properties required in it (Suzuki et al., 2019).

As a natural protein-based material, silk has been used for a great variety of biomedical applications, due to its biocompatibility and biodegradability, factors that have a great deal of importance when regarding the healing process and avoiding immune system rejection. The properties of this biomaterial, such as its malleable shape, mechanical strength, and controllable degradability make it the new hot topic for state-of-the-art bioengineering research, in addition to this, silk film is easily manipulated and has an almost optical transparency property, making the perfect target for ophthalmologic applications (Luo et al., 2021).

Furthermore, this particular material has been shown to promote injury healing by reducing inflammation, promoting tissue regeneration, providing a protective barrier, and facilitating cell adhesion, and, due to its production nature, does not require as much care, training to manipulate, time consumption, and monetary investment as other biomaterials alternatives, such as the use of amniotic membranes or decellularized membranes (Tran et al., 2018). Additionally, the antimicrobial properties of silk in preventing bacterial growth and biofilm production can help prevent infection in wounds (Chirila et al., 2008).

### 4.1. PEG

On the other hand, PEG a synthetic polymer is mostly known for being widely used in several biomedical applications, due to its ability to improve tissue regeneration and reduce local inflammation, by preventing dehydration and enhancing angiogenesis and collagen synthesis together with increased breakdown of fibrin and dead tissue, on top of being an antibacterial dressing, this way, reducing scar formation (Brown et al., 2019).

### 4.2. Fibroin silk membrane in conjunction with PEG

When used in combination, fibroin, an isolated silk protein, treated with PEG has shown interesting synergetic results, fibroin membranes when treated with PEG, shows increasing elastic modulus and lowering elongation, while being able to maintain its tensile strength when compared to non-treated ones. While not able to improve cell attachment, enhancement of cell proliferation, and structural properties optimization were noted, making this particular advance stand out in the ophthalmologic community as a whole, seeing as this would improve, by a long shot, the usage of biomaterials to promote corneal healing in an accessible way to the population (Suzuki et al., 2015).

Overall, the synergic utilization of PEG and Silk membranes in wound healing has the potential to be the next big deal in terms of faster recovery and reduced scarring, especially in life-threatening situations, and as research in this area continues, even more applications for these materials will likely surface, further expanding their potential to unknow applications for patients in need (Suzuki et al., 2015).

The obtention of cells cultures derived from primary tissues is a valuable tool for testing and investigating potential therapies, biological processes, and diseases mechanisms, unfortunately, primary cells have limited proliferative capacity, as well as bad adaptability to not physiological ambient, turning the long-term studies extremely more limited and complicated (Ghallab, 2013). To overcome this barrier, usually, the primary cells are immortalized by the use of viral vectors, allowing them to have a higher proliferative capacity, as well as adaptability, making the study life and reproducibility increase (Janik et al., 2016).

Obtaining an immortalized cell culture that is derived from a primary structure involves several steps. Firstly, the primary cells are surgically separated from the tissue and cultivated in a suitable growth medium, followed by the addition of a viral vector carrying SV40 large T antigen into the culture. This process results in an SV40 large T antigen junction and subsequent inactivation of factors and proteins such as p53 and Rb, both playing a crucial role in cell cycle regulation, this inactivation of apoptotic events will lead to the immortalization of the culture (Linder; Marshall, 1990).

To further investigate the corneal wound in *vivo* models, rabbit corneal structure is widely used in the ophthalmologic field, and may help in further attempts to correctly recreate an in vivo model of the wound, and follow its regeneration and efficacy when regarding visual loss in the wound healing process when supplemented by a variety of biomedical procedures and biomaterials (Eren et al., 2016).

In conclusion, the extracellular matrix, intercellular junctions, growth factors, and cytoskeleton arrangement play critical roles in maintaining the integrity and function of corneal epithelial cells. And as such understanding, characterizing, and quantifying these mechanisms that regulate this process is essential for developing effective treatments for corneal disorders or injuries and optimizing the correct healing process.

# 2. Hypothesis and objectives

Therefore, the hypothesis of the following TFM is that the usage of Fibroin Silk membranes with 5%PEG (SF-PEG) could provide a better alternative substrate than the usage of the present biomaterials when regarding corneal wound regeneration on an *in vitro* model with corneal Epithelial cells.

# 2.1. Specific objectives

1. To study the behavior of immortalized corneal epithelial cells growing on SF-PEG membranes and compare them with cells grown on a physiological ECM component of, collagen I. Time to reach cell confluence, morphology, and characteristic markers expression.

2. Assess the capacity to react after an injury inflected in a confluent culture grown on both subtracts and compare the speed of closure of the wound.

3. Describe the mechanisms involved in the wound closure process: Proliferation and migration.

4. Display the agents involved in this migration: cytoskeleton and focal adhesions.

# 3. Materials and Methods

## 1. Preparation of cells culture

The cells culture presented in this work were HCE SV-40 immortalized human cornea epithelial cells, kindly provided to us by the Grupo de Biomateriales del Ins. de Ciencia y Tecnologia de Polimeros -CSIC.

All cell cultures were made on 35mm plates, using the Dulbecco's modified Eagle's medium and Ham's nutrient mixture F-12 (DMEM-F12 Gifco) cell culture medium with 1% antibiotic-antimycotic solution (Sigma), and 10% fetal bovine serum FBS; (Sigma; St. Louis, MO, USA). Those that were subject to wounding were cultivated in a Serum-Free medium, to not have the possible growth factor presented in bovine serum to misrepresent the healing process concerning time, cell proliferation, migration, growth factors secreted, and cellular signaling.

The initial cell sample was composed of a concentration of 200.000 cells in a 1 mL sample of serum-free medium (SFM) and was added to all samples in a total volume of 2ml per plate. All the cellular procedures were done inside a sterile laminar-flow hood HeraSafe (Heraeus, Germany) were incubated in a CO2 chamber (Hera cell (Heraeus) at 5% $CO^2$ and 37ºC for 48 hours to achieve full confluency.

## 2. Silk Membrane (PEG5%) Preparation

The membranes were provided to us by the CSIC team, 2,6% silk fibroin and 4,3% PEG (300mW) in a plate of 90mm, and have to be submerged in 200mL of MiliQ water for 24 hours, and are stored at $4^0$C.

Afterward, they are punched into a 35mm sub-sample, to fit the plate used in cell culture, and finally fixated on the bottom with sterile ring-shaped support, to prevent cell migration and membrane translocation.

All membrane manipulations and procedures were made in a sterile laminar-flow Hood.

## 3. Collagen 1 subtracts preparation

The substrate enrichment with collagen was diluted from a commercialized standard sample from Corning, which had a concentration of 3.4mg/mL of collagen one, utilizing 2mL of the said sample and diluting in 8mL of MiliQ water, making a -stock solution of 10mL with a concentration of 680ug/mL

To recover the plate, 90µl of the stock solution was added and further diluted in 1.91mL of MilliQ water, the samples were later sealed with parafilm and incubated for 24 hours at $4^0$C and without exposure to light.

After this initial incubation, the liquid contents were discarded, and all of them were exposed to U.V. light for 20 minutes. After the exposition to U.V., the plates were sealed with parafilm until usage and stored at $4^0$C and without exposure to light. All sample manipulations and procedures were made in a sterile laminar-flow hood

## 4. Scratch Wound

All wounds were made on plates that presented full epithelial cells confluency using an iron tool with a ball-shaped head with the dimensions 9 from RE+G, striving for a standardized wound longitude and severity.

To make the wound, the medium used to achieve confluence was discarded, and in the center of the plate, the initial point of the wound was marked with a pen on the lateral of the plate, to have a proper interpretation of the upper (UP), Middle (MD), and Down (DW) portion of the same. After the wounding, the sample was washed with 1mL of PBS and then 2mL of the medium was added to each plate.

## 5. Wound and regeneration follow-up

Samples and their respective injuries were registered as soon as they were made, our sample size consisted of 5 tissue cultured dishes for each substrate and were differentiated by wound induction time: (0 hours), after 24 hours of incubation, and after 48 hours using an $_X$20 magnification and Leyca inverted microscope (Model DMIL LED Fluo) and utilizing Leyca FLEXACAM C1 camera and Picture analyzing software. To properly follow the healing process as a whole, the wound was subdivided into Upper (UP), Middle (MID), and bottom (BOT) subparts, and pictures of each part were registered and analyzed individually from the injury to the close, meaning that each substrate, at each specific time, had 3 photos taken of the wound to further analyses. healing follow-up as a whole until fully closed. In the end, there were a total of 280 photos taken from samples, 140 of the collagen substrates and 140 of SF-PEG, these 140 pictures were distributed in the following order: 20 pre-wounds, 40 at 0 hours, 40 at 24 hours, and 40 in 48 hours.

Pictures were later analyzed ad quantified by the FIJI 1.53t Image J analyzing software.

## 6. Cell characterization protocols and procedures

The samples were further subdivided into wounds that were fully healed (48 hours) and only partially healed (24 hours), in both cases, fixation was accomplished using paraformaldehyde at 4% for 10 minutes. The samples were then submersed in 2 mL of PBS, sealed with parafilm, and stored at $4^0$C and without exposure to light until usage.

The protocols of Immunohistochemistry present in this TFM were FAK (Ref.A-17, sc-577. Sta. Cruz Biotechnology), Cytokeratin 12(Santa Cruz, sc-515882) and Phalloidin Alexa fluor 488(A122379_300U, Invitrogen) and Integrin α5β1(MAB1969, Millipore).

### 6.1. α-SMA Detection

The fixated samples were washed with PBS, followed by permeabilization with Triton X-100 at 0,1% and -$20^0$C for 10 minutes, wash again with PBS, and then blocked with 200µl of Goat serum 3% in PBS for 30 minutes at room temperature. Followed by the addition of 150 µl of a mix composed of 149,4ul Goat serum 3% PBS and 0,6ul of $1^0$Antibody mouse monoclonal anti- αSMA of human origin Dako ready-to-use (1:250 dilution) and left overnight in a humid chamber at $4^0$C.

After leaving overnight wash the sample with PBS for 5 minutes and repeat this process 3 times. For the next step, add 200ul of a mix containing 199ul of PBS and 1ul of $2^0$Antibody Alexa Flour 594 Goat anti-mouse A-11032, Life Technologies (1:200 dilution) and incubate for 2 hours at room temperature and without the presence of light. Afterward, 200 µl of DAPPI was added for 10 minutes and left at room temperature, followed up by another wash with PBS for 5 minutes, and repeat this process 3 times. Finally, a drop of Vectashield (Vector Laboratories, Burlingame) was added to the sample.

### 6.2. Cytokeratin 12 Detection

The fixated samples were washed with PBS, followed by permeabilization with Triton X-100 at 1% for 45 minutes, wash the sample with PBS for 5 minutes, and repeat this process 3 times. Blocked with 200µl of Goat serum 5% in PBS for 30 minutes at room temperature. Followed by the addition of 150 µl of a mix composed of 148,5ul Goat serum 5% PBS and 1,5µl of $1^0$Antibody mouse monoclonal anti-cytokeratin 12(. Sta. Cruz Biotechnology) (1:100 dilution) and left overnight in a humid chamber at $4^0$C.

After leaving overnight, leave the sample at room temperature for 20 minutes, and wash the sample with PBS for 5 minutes, 3 times. For the next step, add 200ul of a mix containing 198ul of PBS and 2µl of $2^0$Antibody (. Sta. Cruz Biotechnology) Goat anti-mouse (1:100 dilution) and incubate for 2 hours at room temperature and without the presence of light. Afterward, 200 µl of DAPPI was added for 10 minutes and left at room temperature, followed up by another wash with PBS for 5 minutes, and repeat this process 3 times. Finally, a drop of Vectashield (Vector Laboratories, Burlingame) was added to the sample.

## 7. Cell migration protocols and procedures

## 7.1 Focal Adhesion Kinase (FAK) Detection

The fixated samples were washed with PBS, followed by permeabilization with acetone at -$20^0$C for 10 minutes, wash again, and then blocked with 200µl of Goat serum Invitrogen 3% in PBS for 30 minutes at room temperature. Followed by the addition of 150 µl of a mix composed of 149,4ul Goat serum 3% PBS and 0,6ul of $1^0$Antibody rabbit polyclonal A17( Ref.A-17, sc-577. Sta. Cruz Biotechnology) (1:250 dilution) and left overnight in a humid chamber at $4^0$C.

After leaving overnight, wash the sample with PBS for 5 minutes 3 times. For the next step, add 200ul of a mix containing 199ul of PBS and 1ul of $2^0$Antibody Alexa Flour 594 Goat anti-rabbit A-11037, Life Technologies (1:200 dilution) and incubate for 2 hours at room temperature and without the presence of light. Afterward, 200 µl of DAPPI(Sigma)was added for 10 minutes and left at room temperature, followed up by another wash with PBS for 5 minutes, and repeat this process 3 times. Finally, a drop of Vectashield (Vector Laboratories, Burlingame) was added to the sample.

## 7.2. β actin Detection

The original commercial solution flask of Phalloidin (Invitrogen Alexa Fluor 488) was dissolved in 150µl of DMSO, obtaining a stock solution of 2.000 assays/mL, of said sample, 5 samples of 1µl are made and stored at – $20^0$C without the presence of light until further use.

The fixated samples were washed with PBS, followed by permeabilization with Triton X-100 at 0,1% for 30 minutes, wash the sample with PBS for 5 minutes, and repeat this process 3 times. A mix utilizing 0.5µl of the DMSO-Stock solution diluted in 200µl of PBS is incubated in each sample for 30 minutes without the presence of light. Afterward, 200 µl of DAPPI was added for 10 minutes and left at room temperature, followed up by another wash with PBS for 5 minutes, and repeat this process 3 times. Finally, a drop of the mix of Glycerol-PBS (60% Glycerol-40%PBS) was added to the sample to be analyzed.

The entirety of the samples was imaged in confocal Leica TCS SP5X and Leica TCP SPE.

Software Leica LAS AF 2.3.6.

## 8. Proliferation Assays

The cell cultures with and without collagen subtract were made on 13mm diameter 96--well cell culture dish using the DMEM-F12 (without red phenol) cell culture medium with 1%*antibiotics, in a Serum-Free medium, to not have the possible growth factor presented in bovine serum to misrepresent the healing process concerning time, cells proliferation, migration, growth factors secreted and cellular signaling.

The initial cell sample was composed of a concentration of 20.000 Cells in a 100 µL sample of Serum-Free medium and was added to all samples in a total volume of 200 µl per dish well.

The cell cultures with membrane were made into a 35mm sample cell culture dish diameter, using the DMEM-F12 (without red phenol) cell culture medium with 1% antibiotics, in a Serum-Free medium, to not have the possible growth factor presented in bovine serum to misrepresent the healing process concerning time, cells proliferation, migration, growth factors secreted and cellular signaling.

The initial cell sample was composed of a concentration of 200.000 cells in a 1 mL sample of Serum-Free medium and was added to all samples in a total volume of 1 ml per dish well. All the cellular procedures were done inside a sterile Laminar-flow Chamber. After the inoculation, samples were incubated in a CO2 chamber (Hera cell (Heraeus) at 5% $CO^2$ and 37ºC for 48 hours to achieve full confluency.

All wounds were made on plates that presented full epithelial cells confluency using an iron utensil with a ball-shaped head with the dimensions 5 from RE+G, striving for a standardized wound longitude and severity.

The samples were then separated into 3 groups, based on the time in which the initial wound was inflicted, those with wounds that last 24 hours,0 hours, and pre-injury, after they achieved confluency. The membrane samples were then punched with a 6,0 mm Biopsy Punch from Stiefel in order to fit into the 13mm diameter 96-well cell culture dish. All samples medium were then discarded and 200 µl of DMEM-F12 (without red phenol) cell culture medium with 1% was added.

Afterward, 20 µl of WST-1 solution was added per well, the samples were then incubated for 1:30 in a in CO2 chamber (Hera cell (Heraeus) at 5% $CO^2$ and 37ºC. They were then shaken thoroughly for 1 min. The absorbance was measured, in both of the treated and untreated samples using a multiskan FC Thermo-fisher plate reader at 450-620 nm.

## 9. Scanning Electronic Microscopy

The epithelial cells, after reaching confluency on a SF-PEG were fixed in Palay fixative for 24h and washed in PBS three times. Then samples were dehydrated in increasing graded concentrations of ethanol for 10 minutes respectively. After samples were sputter coated with gold for 15 seconds leaving a 4nm coating on samples They were imaged using a **Quanta 200FEG** environmental chamber **SEM** with tipo Everhardt-Thornley (ETD) detector, HIGH VACUUM 15 kV).

## 10. Transmission Electronic Microscopy

The epithelial cells, after reaching confluency on an SF-PEG, were fixated in Palay- by immersion for 72 hours. After the immersion period, the samples were then washed in PBS 0,1M pH 7,4 at room temperature for 10 minutes, 3 times. Following that, re-fixation in $O_SO_4$ at 1% in PBS for 2 hours with constant agitation. The samples were washed with distilled water for 3 minutes 2 times. After that samples were dehydrated in acetone with a crescent concentration. The samples were immersed in a mixture of 50% propylene oxide and Spurr resin for 24 hours. Selected sections were cut on LKB ultramicrotome (Prodkter Ab, Stockholm Sweden) and examined under a Jeol JEM-1011HR electron microscope (Jeol Ltd., Tokyo, Japan) of the Microscope Unit of the *Parque Cientifico-Universidad de Valladolid*. Photomicrographs were obtained with the ES1000W Erlangshen CCD Camera (Gatan Inc., Pleasanton, USA).

## 11. Statistical Analyses

The statistical analysis, as well as the graphical representations, were done on GraphPad Prism 8.02.

After all, the data was collected, there was a pre-analysis, as well as the exclusion of all the outliers on every sample. This process was done by adjusting the exclusion of outliers to 10% and testing the normality of each sample right afterward. For the variables that had a normal distribution, the

tests made were ANOVA and t-test, to correctly interpret the final results of the experiment, when the data had a non-normal distribution, the tests used were the Mann-Whitney test and Kruskal-Walli's test. A p-value of ≤ 0.05 was considered statistically significant.

# 4. Results

## 1. Human Epithelial Cell Confluency on different substrates

As it's possible to see that although there is no significant difference between the Membrane and Collagen 1 samples, they were both significantly different than the non-supplemented cultivate (Image1.), making it clear that subtract supplementation has a significant impact on in vitro cell cultivation and reproduction.

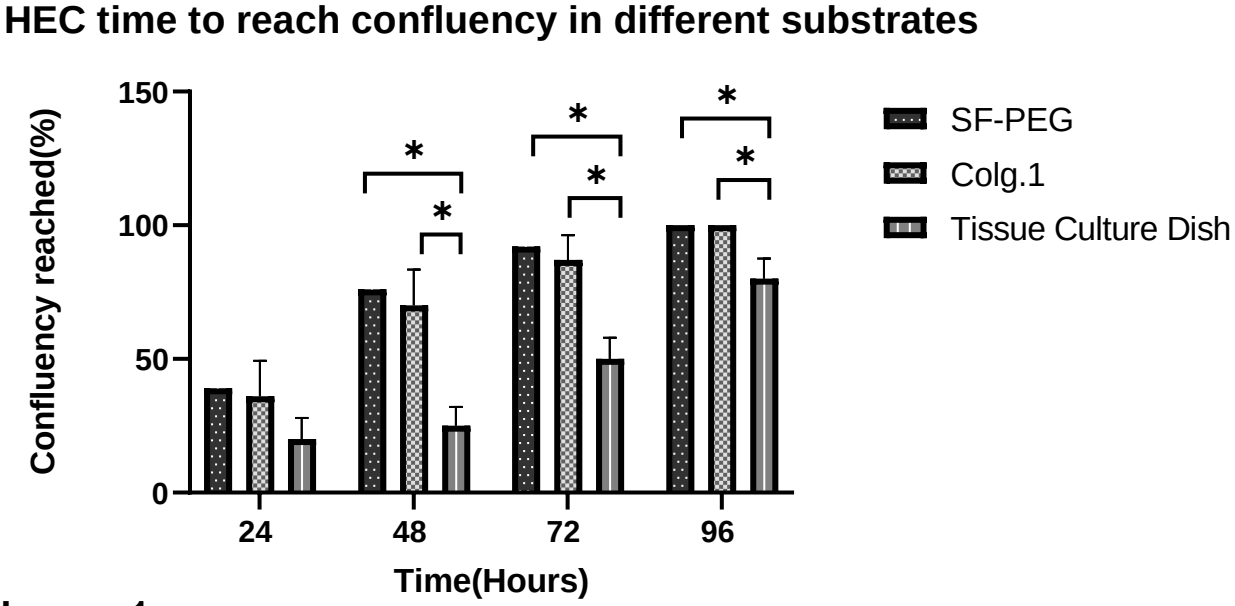


**Image 1**. Comparison between time to reach confluency of Epithelial Cells on Collagen. 1(Colg.1); Membrane samples (SF-PEG) and tissue culture dish. The error bars represent Standard deviation. *=p<0,05.

## 2. Wound Regeneration on SF-PEG and Collagen 1 subtracts

After the initial wound, the samples had a similar time of closure, in both substrates, the wound was fully healed in 72 hours, and by 48 hours after injury infliction, all had at least 97% of closure (Image 2.). Because of this, we separated the samples into pre-wound, 24 hours, and 48 hours, to correctly differentiate the healing process. The analysis of the wound area(Image 3.) demonstrates that wounds on the membrane tend to be bigger than in collagen. Despite this fact, the SF-PEG substrate was able to fully regenerate at the same time spawn as the collagen samples (Image 4.).

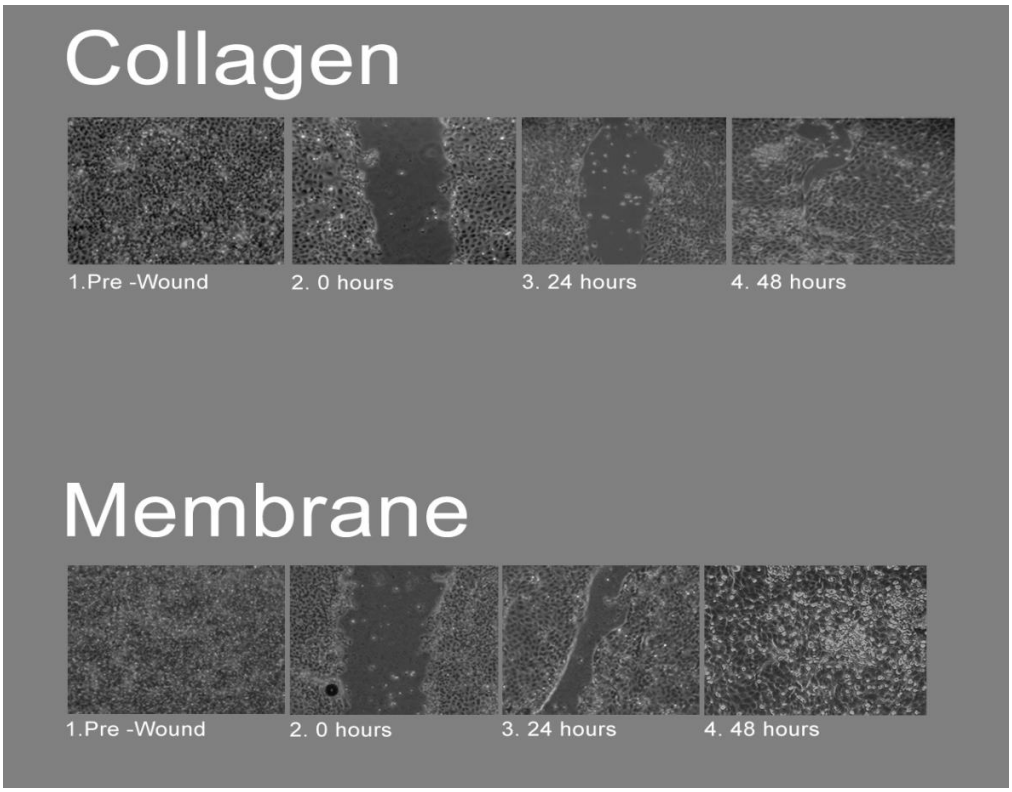


**Image 2.** Collagen and membrane samples on the time intervals and recovery rate of each one.

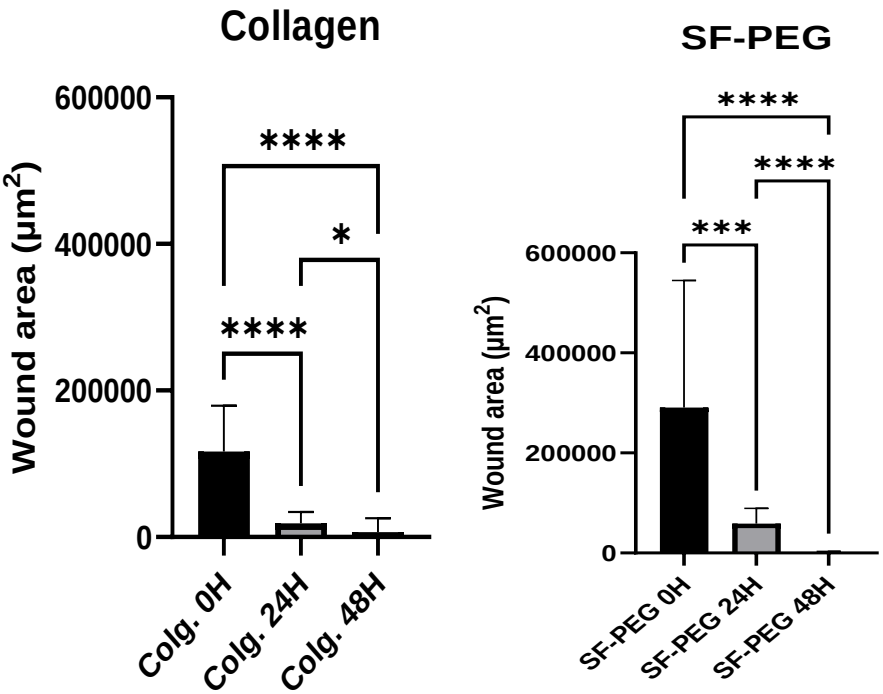


**Image 3.** Collagen and membrane samples on the time intervals and recovery rate of each one. The error bars represent Standard deviation. *=p<0,05; ****=p<0,0001.

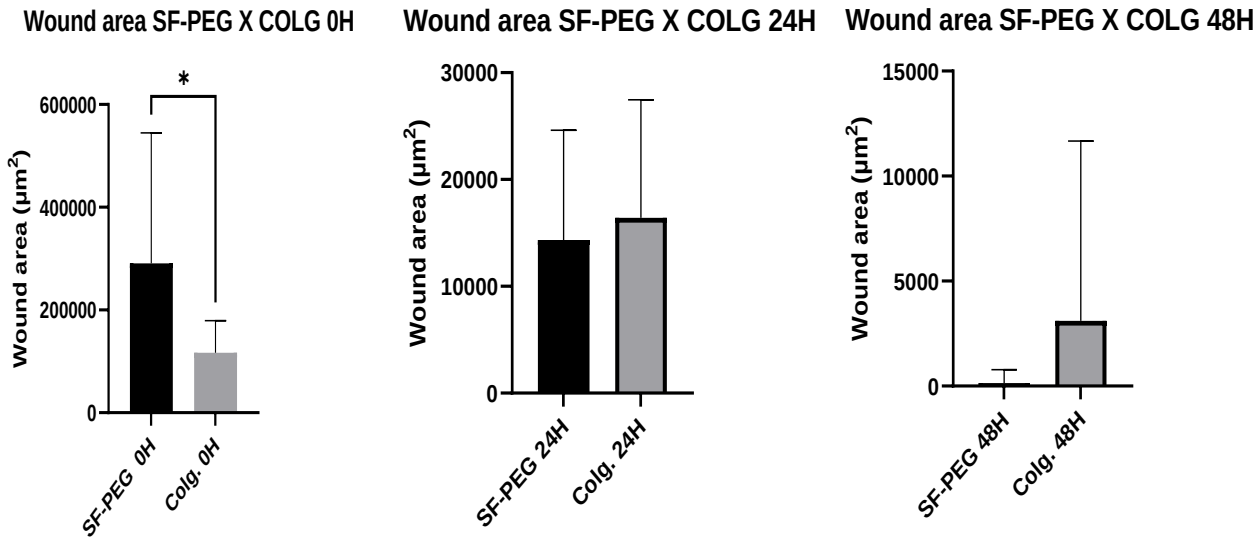


**Image 4.** Analyzing the significance between the area of the wound of the epithelial cells on each substrate, Collagen (COL.) and Membrane (SF-PEG), in the function of the time. The error bars represent Standard deviation. *=p<0,05.

## 3. Cellular Characterization

### 3.1 CK12 Expression

It's possible to see that the characterization of CK12 was present, in both substrates, suggesting that these cells did not suffer any transformation process that impossibilities the presence of this marker, at any time, being that before or after wound induction and in both substrates (Image 5.1.) (Image 5.2.).

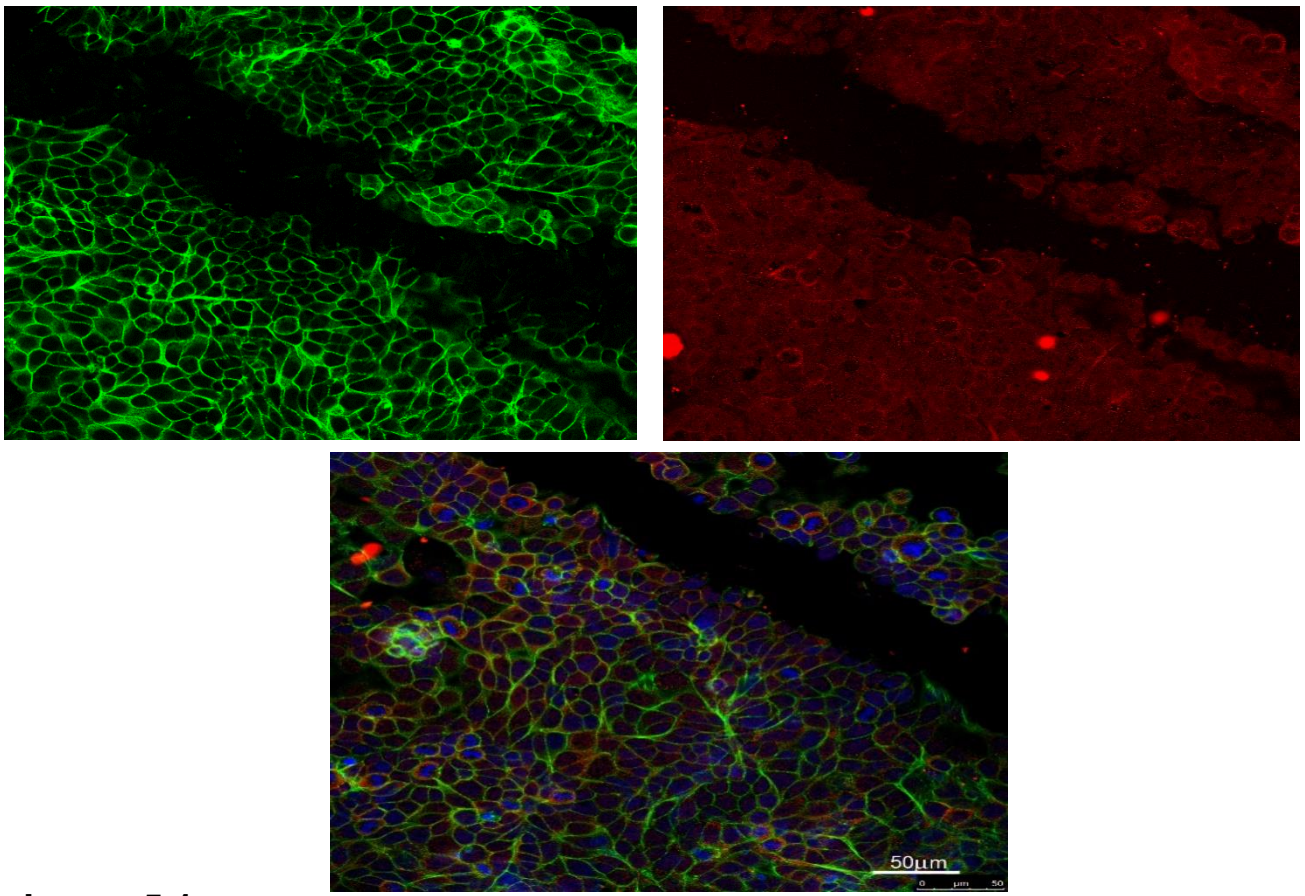


**Image 5.1.** Imnuhistoquemistry of Ck12(red) with Phalloidin (Green) and DAPPI(Blue), on a membrane 24 hours after wound induction.

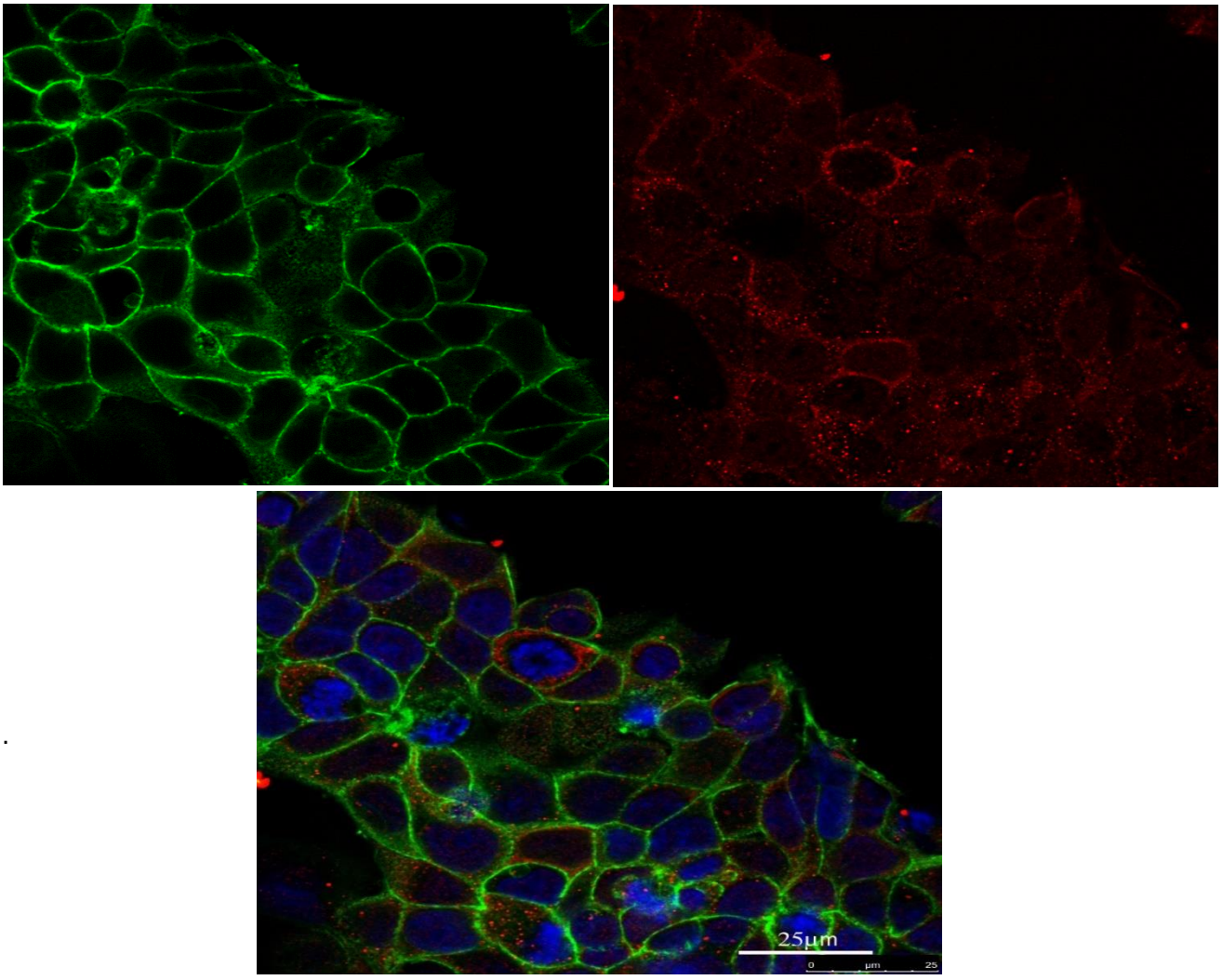


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**Image 5.2.** Imnuhistoquemistry of Ck12(red) with Phalloidin (Green) and DAPPI(Blue), on a membrane 24 hours after wound induction, with emphasis on individual cell characterization.

## 3.2. α-SMA – β-Actin/Phalloidin

As we can see in the Imnuhistoquemistry, all the cell cultures, independent of the substrate or time after wound, and even after wounding itself, have a positive presence of α-SMA (Image 5.3.) (Image 5.4.)

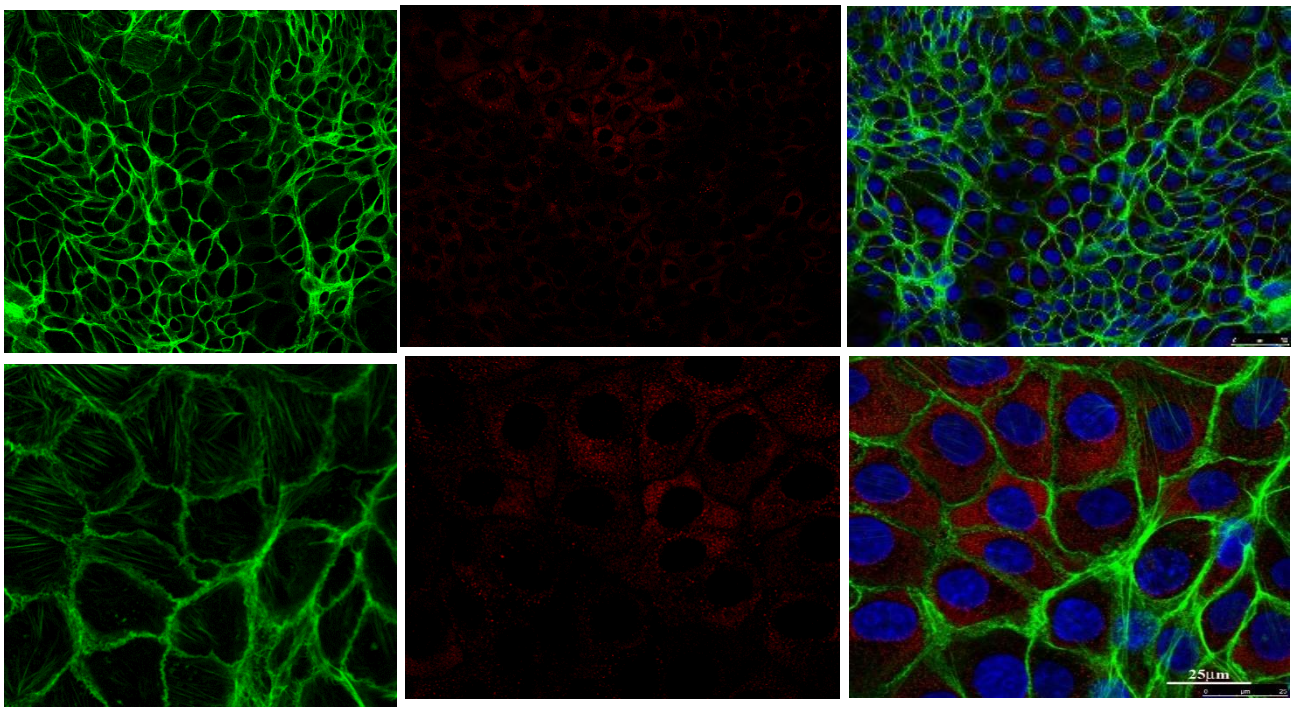


**Image 5.3.** Imnuhistoquemistry of αSMA (red) with Phalloidin (Green) and DAPPI(Blue), on a membrane 24 hours after wound induction, with emphasis on individual cell characterization and full panoramic view.

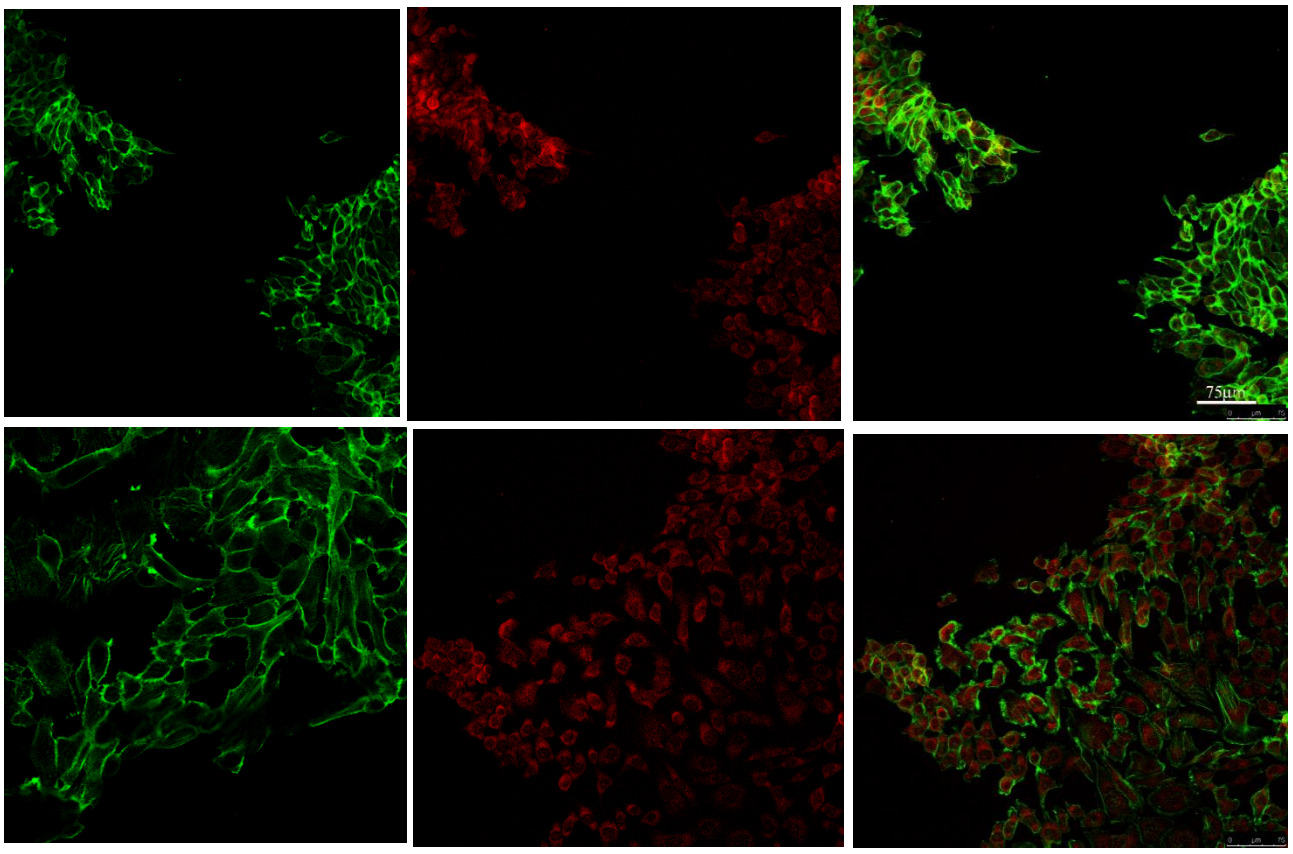


**Image 5.4.** Imnuhistoquemistry of αSMA (red) with Phalloidin (Green), on a collagen-enriched substrate 24 hours after wound induction, with emphasis on individual cell characterization and full panoramic view.

## 3.3. FAK– Phalloidin (β actin expression)

It's possible to see the presence of FAK on all samples, independent of substrate growth, time of wound, and even presence of a wound, indicating that the adhesion process in both collagen substrate and membrane was possible (image 5.5.) (Image 5.6.), making the anchoring adequate in both of the substrates. In both substrates, close to the wound, the cells show elongated cytoplasm, stress fibers, and focal adhesions.

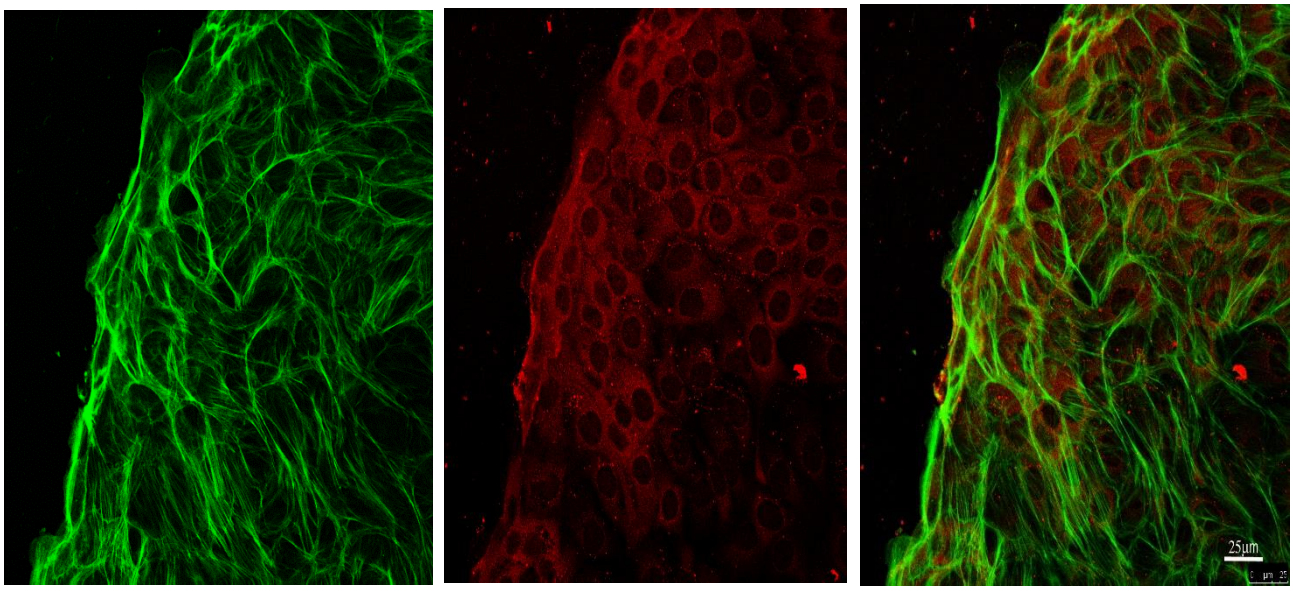


**Image 5.5.** Imnuhistoquemistry of FAK (red) with Phalloidin (Green) on a membrane 24 hours after wound induction, with emphasis on individual cell characterization.

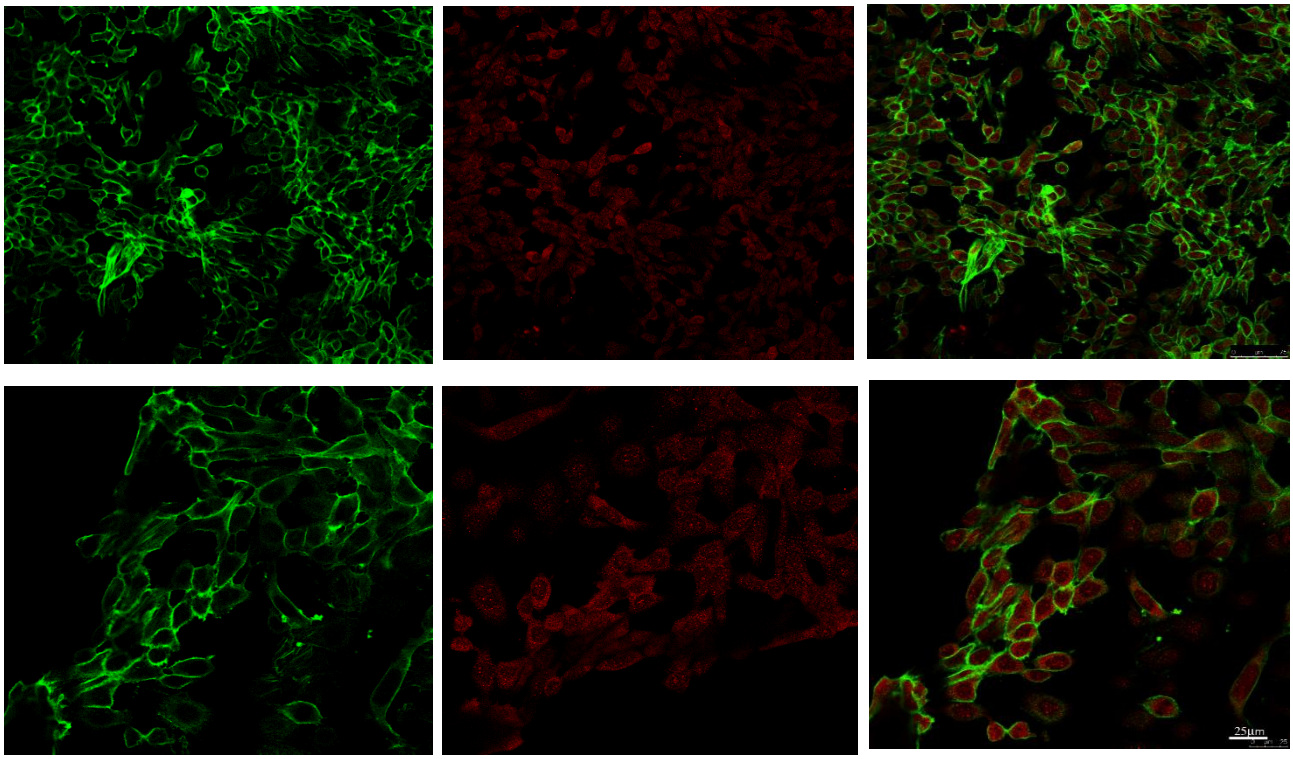


**Image 5.6.** Imnuhistoquemistry of FAK (red) with Phalloidin (Green), on a collagen-enriched substrate 24 hours after wound induction, with emphasis on individual cell characterization and

## 4. SEM

It´s possible to notice, the epithelial cells when reached confluency tend to have a polygonal morphology, a tear-like shape nucleus, with numerous nucleoli, signifying great protein synthesis activity, furthermore, higher magnification show tight junctions between the cells (Image 6.1; image 6.2.; Image 6.3).

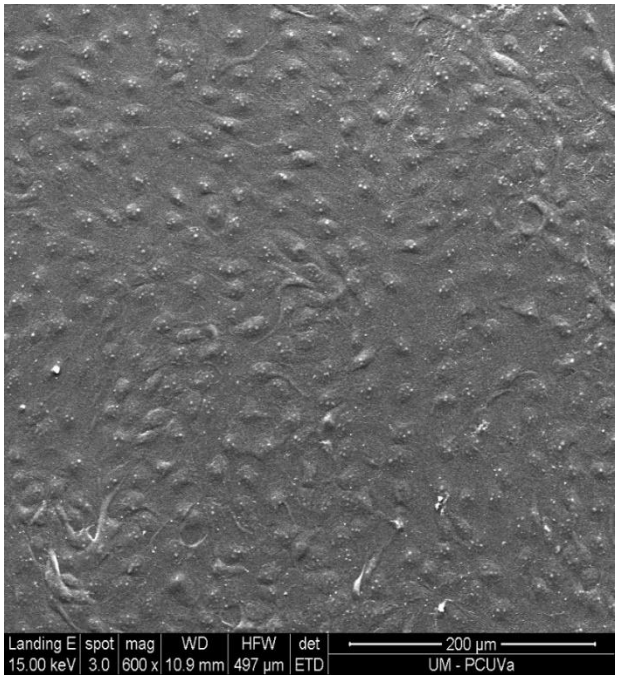


**Image 6.1.** Epithelial cells reaching a confluent state on the SF-PEG membrane- Scanning electronic microscopy (X600).

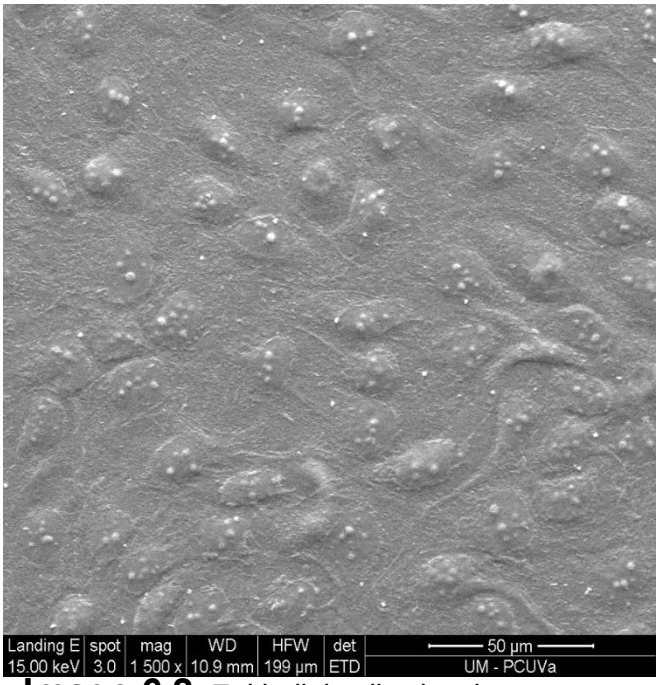


**Image 6.2.** Epithelial cells showing numerous nucleoli and junctions between them when grow on SF-PEG- Scanning electronic microscopy (X600).

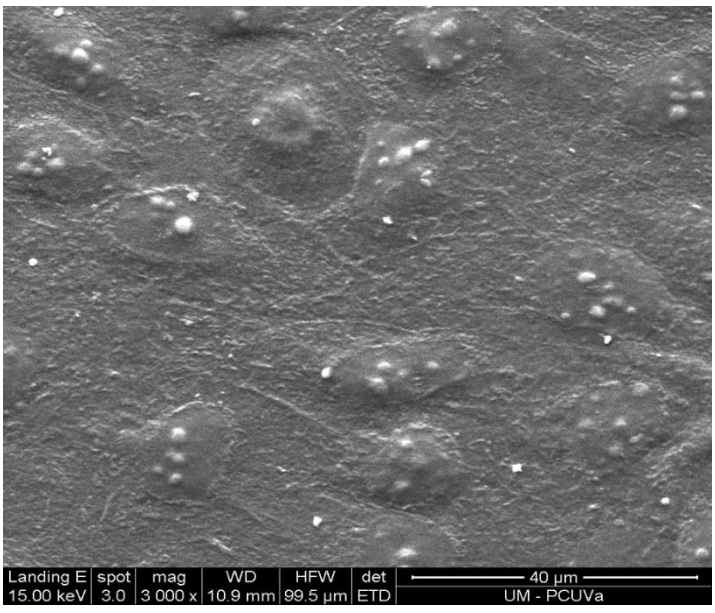


**Image 6.3.** Epithelial cells Nucleol in evidence, showing high levels of protein synthesis in the SF-PEG membrane- Scanning electronic microscopy (X600).

## 5. TEM

It´s possible to notice, the epithelial cells are attached to membrane surface correctly (Image 7.1.), although it is not possible to see specific cells structures that provide stable junctions between the substrate and the cells(Image 7.2.). The metabolic activity of these cells seems to be high, due to the presence of the multiple excretion vesicles noted on the epithelial cells, which seemed to have smaller dimensions than usual (Image 7.3. ). In the apical zone of the epithelial cells tight junctions are present (Image 7.4.).


**Image 7.1.** Transmission microscopy evidencing the Epithelial cell Membrane being attached to the SF-PEG membrane surface

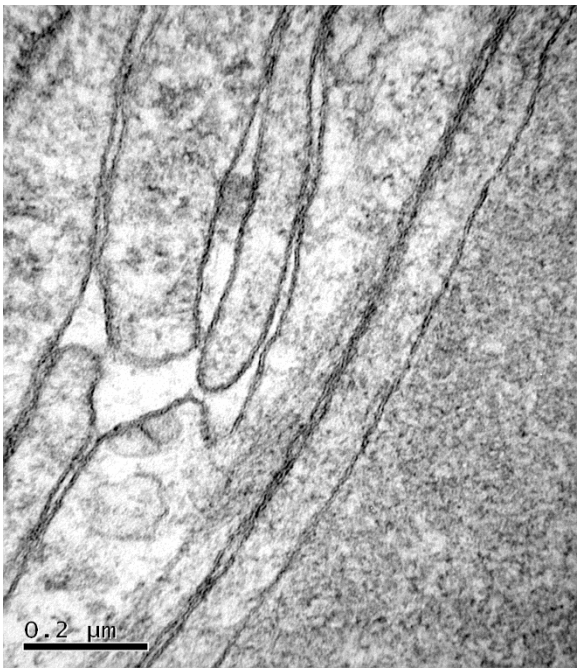


**Image 7.2.** Transmission microscopy evidencing the lack of specialized junctions between the SF-PEG and epithelial cells.

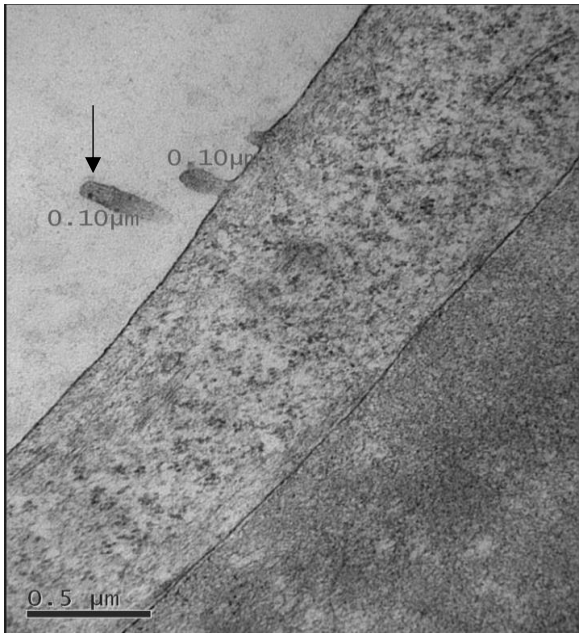


**Image 7.3.** Transmission microscopy evidencing excretion of small vesicles.

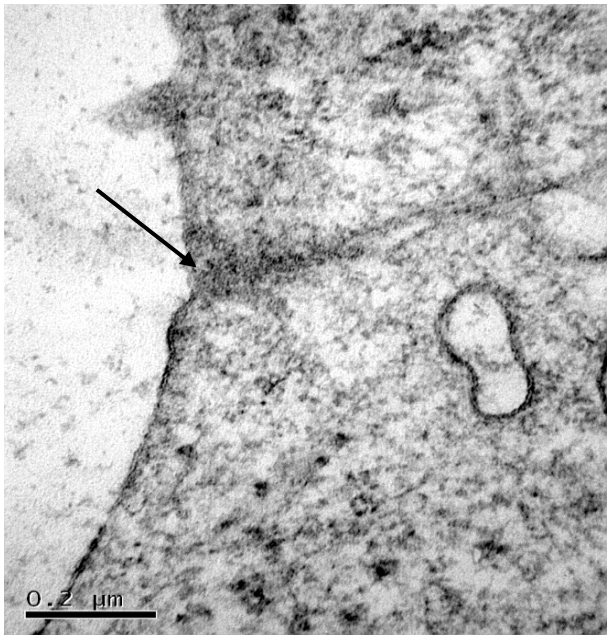


**Image 7.4.** Transmission microscopy evidences the junction between the epithelial cell themselves.

## 6. Cell Proliferation assay

It's possible to see that, although membrane values tend to be lesser than collagen results, both results show clearly that 24 hours after injury, the metabolic activity rises up in a significant manner on both substrates (Image 8.1.) (Image 8.2.), going in favor of the expected behavior based on contemporary literature.

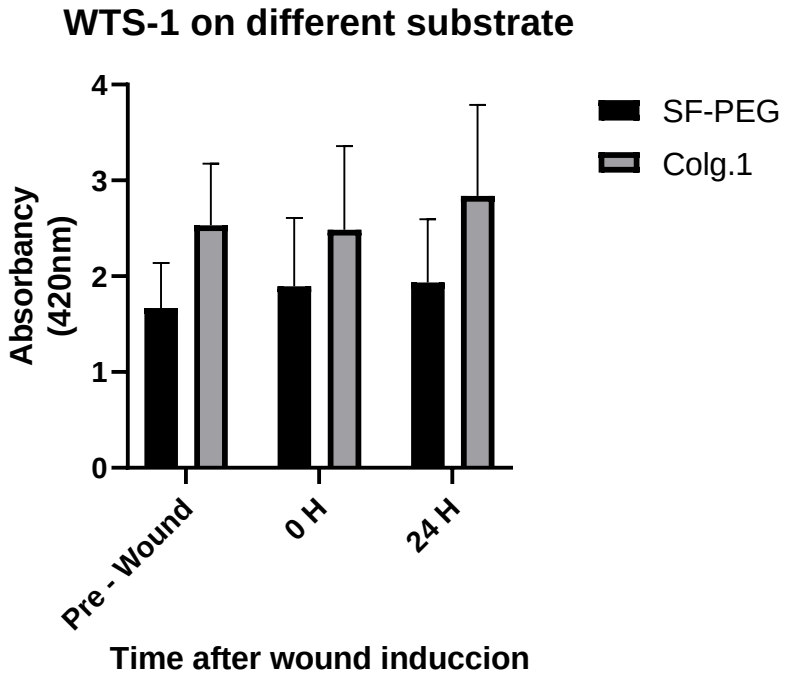


**Image 8.1.** Compared WTS-1 absorbance on membranes and collagen substrates, pre-wound (PH);0 hours after(0H); 24 hours(24H). The error bars represent the Standard deviation.

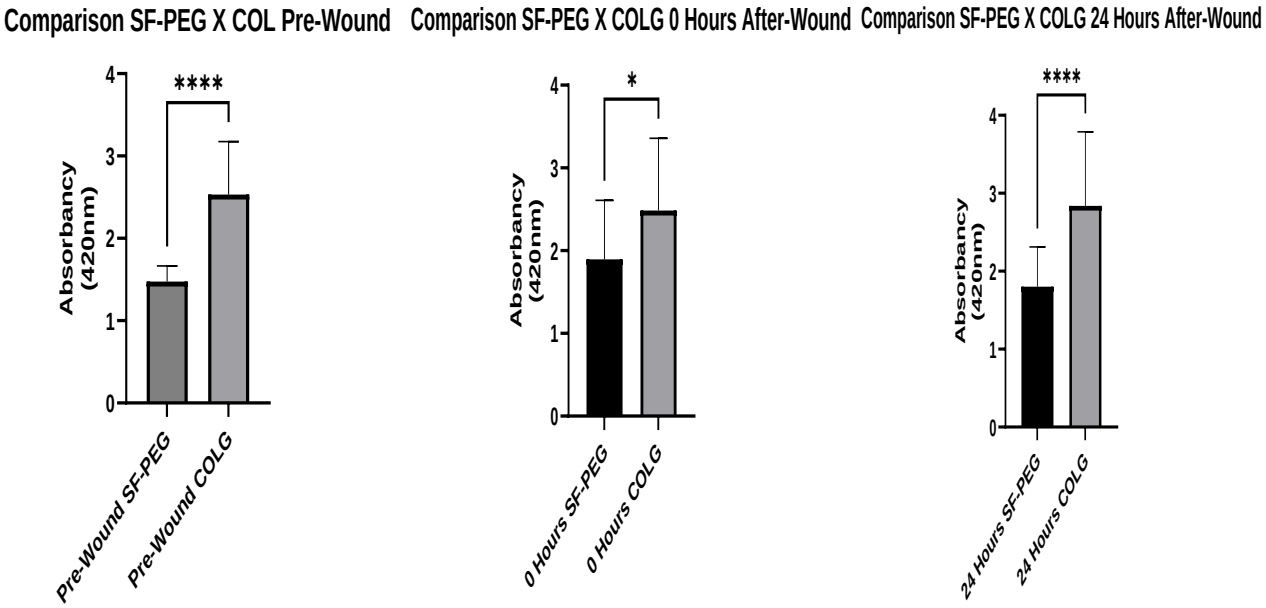


**Image 8.2.** Compared WTS-1 absorbance on membranes and collagen substrates in the function of time, to analyze the differences between metabolic activity in each interval. The error bars represent the Standard deviation. *=p<0,05; ****=p<0,0001.

# 5. Discussion

The results of our study show that the use of the substrates notably improve the confluency time, as it's seen that both membranes and collagen- samples have all reached confluency as soon as 48 hours, while the polystyrene plates had a mean of 25% confluency at this time period, making it clear that both SF-PEG and collagen1- subtracts can significantly shorten the time need to cultivate these cells samples on a serum-free state, as seen in other collagen-based biomaterials(Orwin, E. J. Hubel, A., 2000) and can probably make the process even faster when presented in a serum-rich *in vitro* model.

As it's seen, the collagen 1 samples and Silk membranes had a very similar behavior when regarding healing, and both were significantly different from the regular cell dishes commonly used in cells assays, this could suggest that SF-PEG can have similar, if not better, healing potential than the expected from these cells on its physiological condition and other collagen-based materials (Orwin, E. J. Hubel, A., 2000). This could represent a great step in the usage of this model on *in vivo* models, followed by its possible adoption in clinical usage.

When regarding the cellular characterization of these cells by means of Imnuhistoquemistry, surprisingly, the immortalized cells can transform an epithelial to a myoblast-like phenotype (EMT), especially in response to stress, such as exposure to serum deprivation and/or hypoxia. These transformed cells undergo changes in gene expression and cytoskeletal organization, events associated with a mesenchymal phenotype (Nagamoto et al., 2000).

It's possible to see that Cytokeratin 12 was present in all samples, independent of substrate, but we were unable to successfully see the presence of Cytokeratin 3 in all cells, independent of the substrate, or in any process of pre, wound induction or post-wound induction, because of that we were unable to determine if the process of multiplication was dependent of the physiological substrate, the Membrane, simple the cell lines utilized in this process, our the protocol utilized, for its knowledge that the process of immortalization can change some cell characteristics (Nagamoto et al., 2000). Nevertheless, is possible to see that these cells do retain the expected behaviors, shape, and regenerating properties of normal immortalized epithelial cells, even if some common biomarkers were not present in the lines utilized.

This process of transformation seen in immortalized cells may alter the results under stress scenarios, thus, providing valuable information for the further investigation of cellular response to stress and injuries, especially in the context of corneal wound healing, for this can have profound implications on the development of potential therapeutics and new disease and injury models (Yamasaki et al., 2009).

This basal expression of α-SMA may indicate that the process of viral immortalization of these lines of epithelial cells this biomarker may not indicate an exclusively epithelial wound process as observed in the literature before (Nakamura; Kunihiko., 2003), being that way more similar to the description of (Nagamoto., 2000) presenting a basal expression of it.

When regarding the cellular migration of these cells by means of Imnuhistoquemistry, the FAK expression was basal in either substrate, indicating that way that both were able to provide adequate adhesion and anchor to these HEC, furthermore, when analyzed in conjunction with the phalloidin results, it's possible to see that prolongation all over when a wound is induced, suggesting that cell migration was happening in an increased rate, and although we were not able to prove this by any

cell assay, this suggests a capacity of cell migration on the Silk membrane similar if not greater, than in a physical substrate (Feng, Y et al., 2014).

While the FAK expression was basal in either substrate, indicating that way that both were able to provide adequate adhesion and anchor to these HECs. When analyzed in conjunction with the phalloidin results, it's possible to see numerous prolongations at the center of which stress fibers appear in wound direction all over cells when the wound is induced. This suggests that cell migration is happening at an increased rate, which is demonstrated by the quicker closure of the wound than in a physiological substrate

As it´s possible to notice, the epithelial cells tend to have a similar shape, size, and structure than in other before-seen biomaterials scaffolds when analyzing the SEM results (Li et al., 2015), with high metabolic activity, linked by the very high density of the beforementioned cell structure. Another noticeable feature it's the presence of tightly intercellular junctions, suggesting a higher adhesion to the substrate and adequate anchoring of these cells on the present biomaterial (Baratta, R.O. et al., 2021) (Li et al., 2015).

When regarding the TEM results, the epithelial cells' membranes clearly spread correctly to the membrane surface, although it is not possible to see a structural stable junction between the substrate and the cells, the presence of tight junctions between these cells and the notable increase of metabolic activity, due to the presence of the multiple excretions vesicles, this scenario can be commonly seen in the state-of-the-art biomaterials (Baratta, R. O. et al., 2021). Although one thing that was not present was the specialized junctions usually made between epithelial cells and collagen-based materials, leading us to believe that this process may differ when regarding epithelial adhesion on the membrane.

These extracellular vesicles, seem to be the same as evidenced by McKay et al., 2020, and as the measurements suggest, this means that these extracellular vesicles may contain protein cargo that promotes myofibroblast differentiation, matrix proteins…. Proteomics studies should be carried out to know the full contents of it, as well as how much this insinuates on the study.

At least, when comparing the cell proliferation assay, it's possible to see a significant increase in cell metabolism as early as 24 hours after wound induction in both membrane and collagen samples, indicating that cell proliferation on these substrates does tends to rise in the healing process in order to accommodate the needed changes in this scenario, as seen in other collagen and amniotic membranes on *in vitro* scenarios (Feng, Y et al., 2014) (Baratta, R. O. et al., 2021).

## Limitations.

Taking in consideration the limitations of the present TFM, it's important to regard that, due to the malleable and bad adhesion capacity of the silk membrane, the silicon ring applied to it, can slightly distorted confluency values, for the diminished total dish area, mean also that fewer cells are needed in order to achieve confluency, but the difference between this medium to the control dish sample is significant enough to make this scenario very much unlikely.

Although is very notable the differences between cell proliferation assay values on collagen and membrane samples, this can be explained due to the fact that we were unable to replicate the cultivation of epithelial cells with a membrane in 24 and 96 well dishes, for cells would die when cultivated with the silicone ring on these conditions, and when cultivated without it, proper cell adhesion would not occur because the membrane itself would not properly adhere to the dish well,

and because of this, initial cell concentration on the membrane could not be the same as on collagen samples.

One solution for this could be the introduction of rings made of different materials or another methodology to properly adhere the membrane to the well without the need for any adjacent equipment at all, but due to the nature, and time limit for the time being, we were unable to recreate the proper conditions *in vitro* to properly measure the full metabolic scenario in this assay, as cell proliferation .

Finally, its notable that future studies are needed to correctly determine the viability of this biomaterial in *vivo* models, for its consequences, and methods of manipulation may widely differ from its possible clinical use, but we can conclude, the Silk membrane with 5% PEG has a great potential for the future use in corneal damage as far as epithelial cells are concerned.

# 6. Conclusions

In the present work, the following conclusions were reached:

- The Silk Membrane with 5% PEG was able to be as efficient as a simulated physiological ambient, as far as wound healing capacity goes, and showed promising signs of its use as a biomaterial having the same time to close wounds as other collagen-based materials.
- The epithelial cells have a proper adhesion and anchoring process on the Silk membrane with PEG, as well as properly expressed biomarkers, and seemingly, without suffering any process of transformation directly because of the membrane, as it’s possible to see on successful biomaterials.
- It is clear that epithelial cells grow, reach confluence and close the wound more rapidly than in control scenarios, and this may be due to a significant increase in proliferation and migration in SF-PEG, showing promise for its use in future studies.

# 7. Future Studies

As for the possible direction of this study, it will be wise to first fully characterize these cells before its added to any substrate, to determine, for example, if these truly do not express CK3, and if not, what other biomarkers would be necessary to correctly characterized these cells, to then move on to the full specter changes when added to any substrate.

It would also be interesting to have a quantifiable manner to correctly measure cell migration and proliferation, although we were unable to correctly measure, maybe a Cyquant essay would help determine such values, or other methods which would not depend on fluorescence values.

Other factors such as the measurement of Growth factors related to healing and cell migration and activity, such as EGF and PDGF-BB be the meaning of ELISA, followed by a proper protocol of cell lysis, which was unfortunately unable to be done at the time for the present work.

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