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Analyzing the Growth of Human Epidermis Equivalents in the Absence of Epidermal Growth Factors

Rachael Tiong[1], Amy Lee and Yunlong Jia[#]

[1]Irvington High School
[#]Advisor

ABSTRACT

Bioengineering of human skin equivalent (HSE) organoids to closely replicate real human skin in vitro is being developed for multiple applications in the medical and scientific fields, such as skin grafting for burn patients or researching treatments for Basal Cell Carcinoma (BCC). This study analyzed the effects of growing human skin organoids in-vitro on a cadaver dermis, using a medium with no epithelial growth factor (EGF), over the course of 7 days. Few studies have been carried out to examine the effects of these parameters. Microscopic pictures comparing no EGF, control (with EGF), and human skin were taken, along with measurements of cell density and average thickness of the living epidermal layer. Our found average cornified thickness of the control organoids was 68.6 pixels (about 1.8 x 10^4 um) and 128.8 pixels (3.4 x 10^4 um) for the no EGF group. The human skin had an average cornified thickness of 47.4 pixels (1.2 x 10^4 um). No EGF resembled human skin better in this aspect. However, no EGF lacked a healthy formation of the top layer in the microscopic pictures and was farther in cell density than that of EGF. A significant value between no EGF and EGF for cell density was calculated ($p<0.05$). These results demonstrate that the absence of EGF is a limitation in mimicking human skin as close as possible. As a whole, this study confirms the necessity of EGF in order to create a more accurate HSE organoid.

Introduction

There are three classifications of epithelial-mesenchymal transition (EMT). [1,2] Type 1 is associated with implantation, embryogenesis, and organ development. [3] Type 2 is associated with tissue regeneration and fibrosis. [4] Type 3 EMT occurs in the context of tumor growth and cancer progression. [5] While they all generate an activated mesenchymal cell phenotype, the biological setting for the process is quite different from one another. Nevertheless, the transformation of all types signifies the completion when the mesenchymal cell migrates away from the epithelial layer in which it originated. [6,7]

In this paper, we focus on Type 2 EMT and specifically on the reconstruction of tissue following trauma and inflammatory injury. [8] A typical treatment includes the use of skin grafting. [9] However, regardless of the choice of treatment mechanism, one factor that is quite pivotal is the control of the amount of growth factor involved. [10,11] Often at the inflammation wound site, there is the presence of a high level of active proteases that would cause degradation of the growth factor. [12-14] In this paper, we analyzed the use of epithelial growth factor (EGF) in the application of human skin equivalent (HSE) organoids cultured on human skin.

The epithelial-mesenchymal transition plays a role in wound repair and tissue fibrosis (Figure 1). In order to facilitate the transition, the epithelial cells need to lose E-Cadherin (CDH1). [15] Losing CDH1 results in a decreased expression of desmosomal, tight junction, and cell polarity proteins. [16] When EGF is present, it induces EMT, lead-

ing to decreased levels of key proteins necessary for cell epidermis stratification, thereby hindering the accurate creation of human skin organoids. [17] Therefore, by removing EGF and ultimately limiting the proliferation of epithelial cells undergoing EMT, we hypothesized that the stratification of the organoid might also exhibit favorable results. The results from culturing an organoid without the EGF proved to be an exemplary sample point in comparison to one that does use EGF [18]. To find a good solution to the growth of human skin, we had to start by understanding the effect of the EGF. High-quality EGF is valuable so it is best to find a good trade-off point where good results of organoids are grown with the least EGF used. [19] With this in mind, we grew organoids without the use of EGF and with EGF to use as a comparison. These results were also compared with human skin.

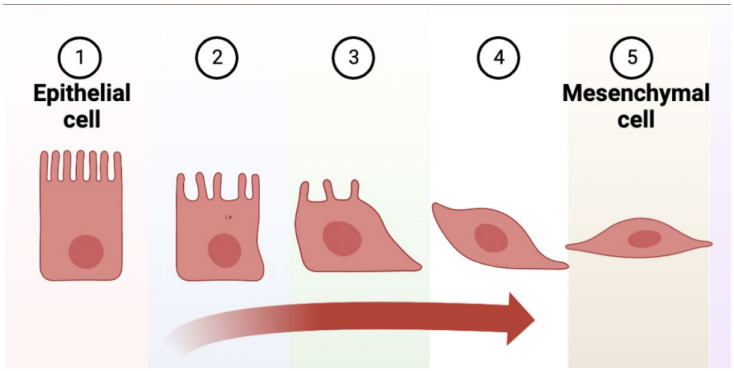

Figure 1, Depiction of the Epithelial to Mesenchymal Transition (EMT)

This figure illustrates the progression of epithelial to mesenchymal cells. On the very left, column 1, shows a fully stratified epithelial cell. This is where cell polarity is defined, with strong cell-to-cell adhesions through specialized junctions, and are restricted to migrate independently, giving the stratified structure. [20] As EMT progresses, the epithelial cells gradually acquire a mesenchymal phenotype with distinct properties. [21] Columns 2 through 4 show a visual of the gradual change in structure from epithelial to mesenchymal cells. [22] At the molecular level, complex signaling networks with molecular regulators, such as transcription factors, growth factors, and extracellular matrix components stimulate the loss of apical-basal polarity and cause cytoskeletal rearrangements. [23,24] The cell-to-cell junctions which are tight and compact are disrupted and allow individual cells to detach from the epithelial layer, increasing mobility. [25] The resulting cellular morphological structure is depicted in column 5 which shows the completed transition to a mesenchymal cell. [26] These cells display a spindle-like morphology and have an elongated shape. [27] Because of the cell shape, they also have increased mobility and enhanced migratory capabilities to move through the extracellular matrix to other surrounding tissues. [28]

Methods

The steps to grow the Organotypic Culture (OTC) used in the growing of organoids are described in Figure 2. First, a cell strainer is placed in each well of a 6-well plate. Take human skin and use forceps to peel away the epidermis from the dermis. Human skin was obtained from discarded surgical samples. Cut the 1.5 cm^2 dermis piece on the strainer, with the top side (non-glossy side) of the dermis facing up. Flip the entire cell strainer containing the dermis upside down. Next, transfer 100ul of Matrigel to the underside of each dermis (Figure 3, 4). Avoid bubbles in the Matrigel. Wait 5 minutes until the Matrigel solidifies and add 4ml of medium without EGF. Prepare 500k to 1 million keratinocyte cells and trypsinize the keratinocytes by placing 2 ml of trypsin in the 10cm culture dish. Place it at 37C for 5 minutes. Quench the 4 ml complete with DMEM media and spin them at 300 X g for 5 minutes to remove the supernatant. Resuspend the cells in 100ul of no EGF medium. Wait for about 10 minutes to let the cells settle down

and attach to the dermis. Place more than 0.5M cells onto the dermis to ensure stratification and differentiation. Slightly and stably move the organotypic cassette to a 37-degree Celsius incubator. Place the organoid into the incubator for 14 days, changing the no EGF media every other day. For the control, the procedure is the same, except after Matrigel, add 4ml of keratinocyte growth medium (KGM) and resuspend the cells after spinning them with KGM. Change the media every other day using KGM [29]. Photographs of the results are shown in Figure 5.

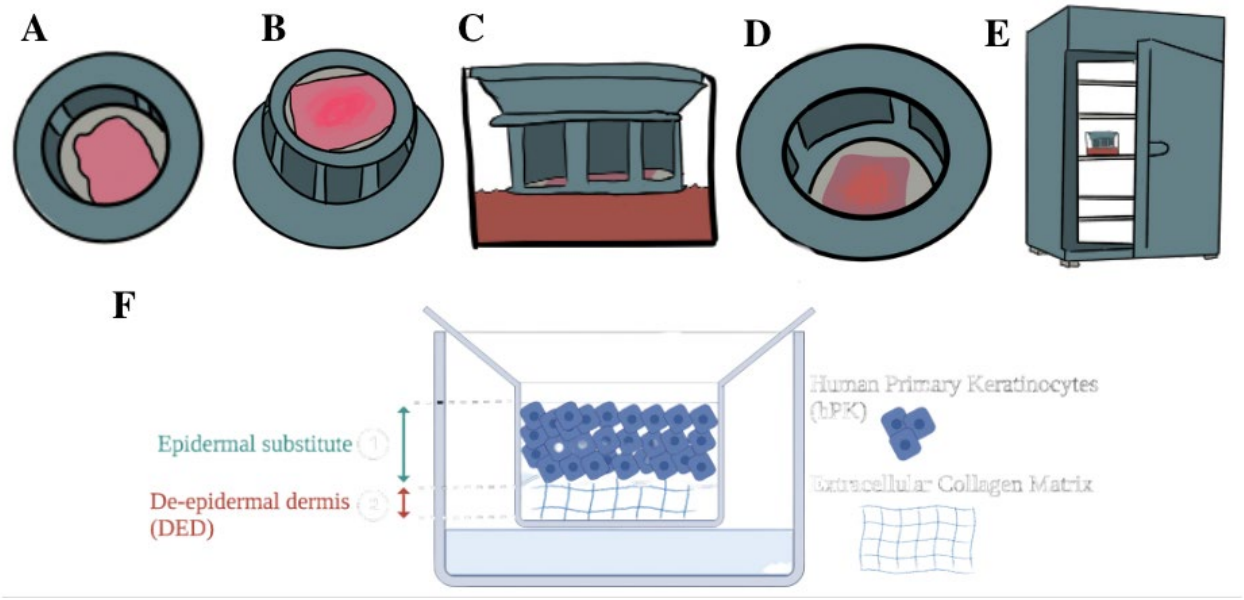

Figure 2. (A-E), Illustration of the procedure in the preparation of the Organotypic Culture (OTC). (F) Illustration of the placement of epidermal dermis in the OTC.

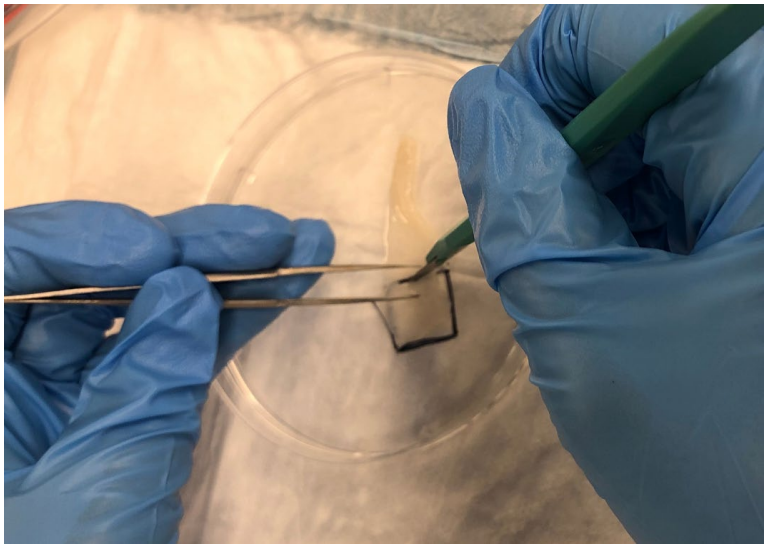

Figure 3. Photograph of cutting the dermis into a 1.5^2 cm piece.

Figure 4. Photograph of organotypic skin culture before incubation.

Results

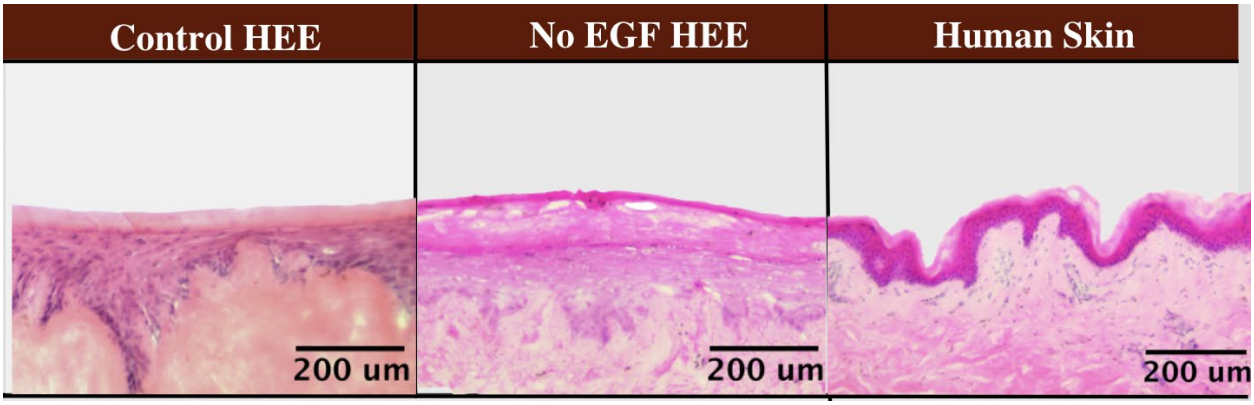

Figure 5. Images of H&E stained samples (10x objective). Organoids are harvested after 2 weeks. The structure of human skin can be observed as ribbon-like in contrast to the organoids. The organoids consist of a flat cornified layer and a curved papillary contact surface with the dermis.

In Figure 5, we see that the human skin has deep ridges at the cornified. The swing of these ridges is considered to be part of the cornified layer. In the No EGF image, the cornified layer is brittle and has small gaps in them. However, the control is much healthier, with a smooth top layer. In addition, it is more clear to see the nuclei of the cells in the control. However, the no EGF seems to have little to no nuclei in the dermis and shows a clear indication that many of the cells are dying. The human skin shown with the least variability in epidermal thickness and the cornified layer thickness is also calculated similarly, having the observed area divided by the length. The results of the measurements are presented in Figure 6.

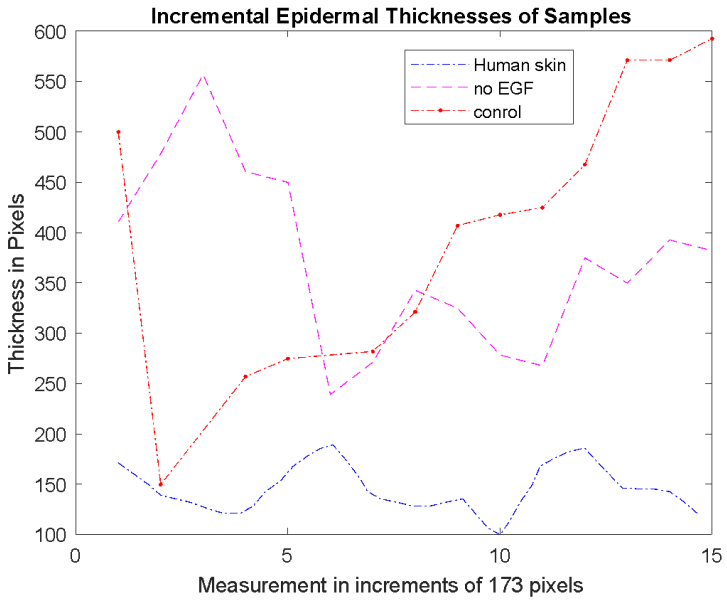

Figure 6. Line graph depicting incremental epidermal thickness of one sample of each condition. Note that the measurements are quantized to increments of 173 pixels and that each pixel is equivalent to 264.38um.

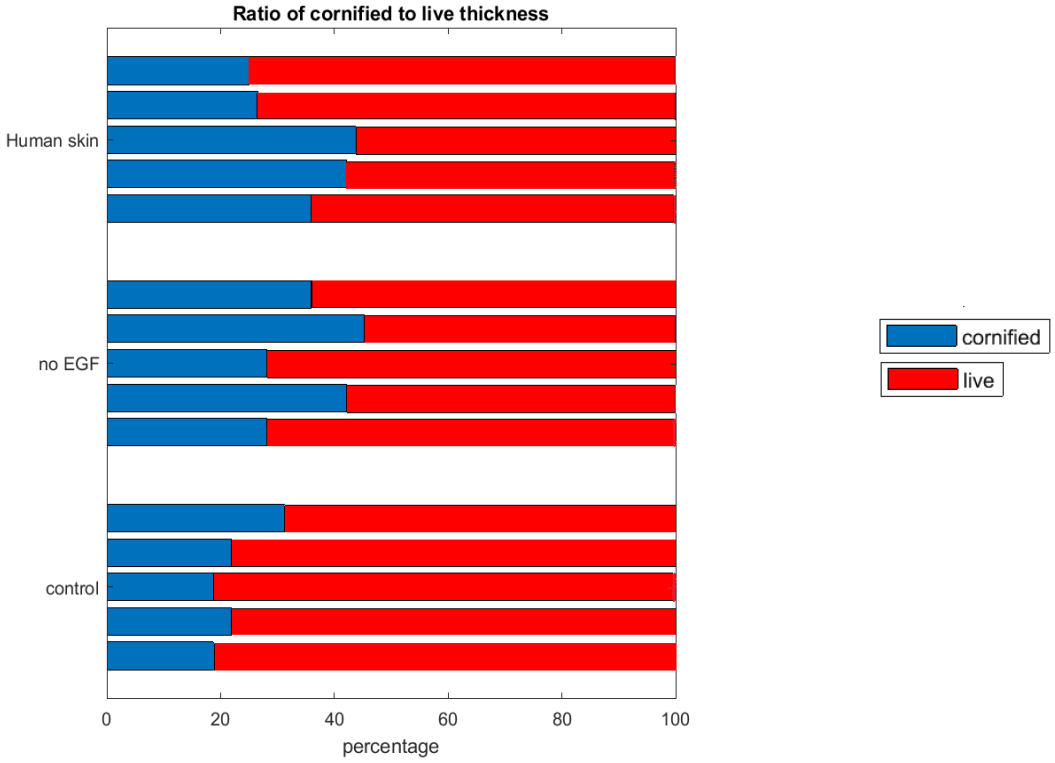

Figure 7. Bar chart showing the ratios of cornified to living thicknesses in the epidermal stratum. The ratios of the no EGF samples more closely resemble those of the human skin. Five samples of each group are used for the measurements.

As for Figures 6 and 7, no EGF does better in the aspect of thickness. The incremental epidermal thickness is not as stable as the human skin, as shown in Figure 6, but no EGF resembles the thickness patterns of human skin more accurately than having EGF. In Figure 7, the ratios of the samples of no EGF to control are more closely related. The cornified layer to the live layer increased, and this was caused by adding no EGF. When comparing it with the control, no EGF does better as it increased ratios slightly higher. This was predicted, as no EGF prevents the rapid transition of cells to undergo EMT. [30] Therefore, by preventing cells to undergo EMT, the structure and stability of the epidermis will remain constant, and cells will continue to keep their rigid structure.

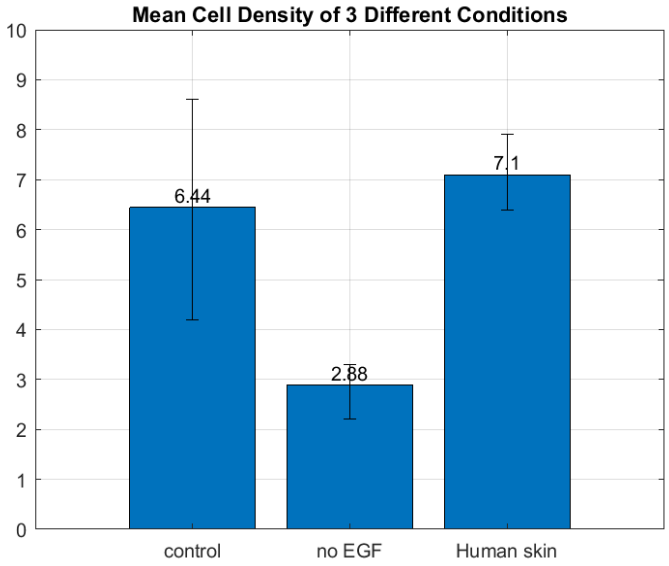

Figure 8. Comparison bar chart showing the mean cell density of the dermis with different development methods. Statistical significance was determined with a t-test between each condition. The procedure to find cell density was to take the average number of cells per square (counted using Fiji cell counter) and divide it by the total volume of the living cell area. [31] This was done 5 times with different organoids of the same groups (5 for control, 5 for no EGF, and 5 for human skin), and was divided by their total to graph the averages. The control's thickness was less accurate as their percentages are lower than actual human skin thickness.

With living cell densities of no EGF organoids considerably lower than that of the control group, removing EGF from the growth of HEE-DED organoids can cause cells to proliferate at a lesser rate compared to the control. This was the goal in the first place: limiting the number of cells transitioning to mesenchymal cells to allow some cells to stay put in the epithelial layer and create a more closely related structure of human skin. However, the control here proves to mimic human skin's cell densities more accurately than no EGF. Density is an important factor as the right cell density allows for optimum cell growth. [32] Lower cell densities result in a lack of cell-to-cell communication, causing slower cell growth and eventually, death. [33] This ties back to Figure 5, as the images show no EGF with barely any nuclei to be seen in the dermis layer. This tells us that the density aspect is an important factor in the creation of human skin organoids and shows that EGF is necessary.

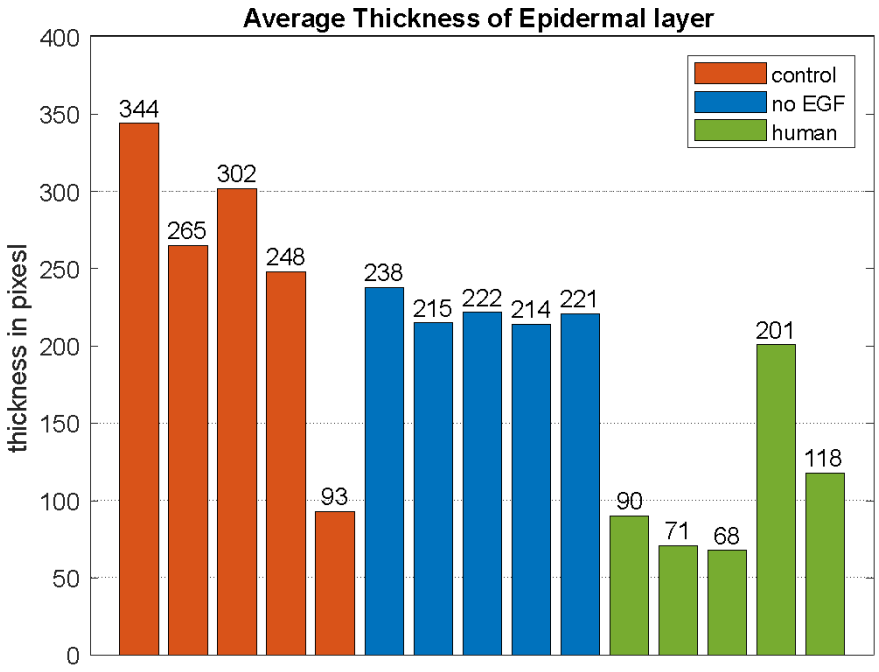

Figure 9. Bar chart showing the average thickness of the living layer between the different conditions. This was done the same way as Figure 6 but with 5 samples in each condition. Notice that the "no EGF" condition shows a more consistent result and in general, has a thickness that is closer to the ideal "human skin" than the "control".

Figure 9 shows another positive result with no EGF through morphology and shape of the organoid. This confirms that no EGF helps with creating accurate thickness in comparison to the EGF control model. The global morphologies of organoids grown with no EGF are more similar to human skin than the control group due to the cornified-to-live thickness ratios (Figures 7 and 9). Our found average cornified thickness of the control organoids were 68.6 pixels (about 1.8 x 10^4 um) and 128.8 pixels (3.4 x 10^4 um) for the no EGF group. The human skin had an average cornified thickness of 47.4 pixels (1.2 x 10^4 um). Therefore, with Figure 9, we can conclude that organoids grown with no EGF more closely resemble human skin in terms of cornified thickness.

So far, there have been some positives with using no EGF but it is not sufficient enough to say that it is the best model for human skin. In fact, there are some drawbacks to completely taking out EGF. In Figure 5, the control (EGF) has a healthier and less fragile epithelial layer. The "no EGF" picture has a less stable and brittle top layer. Visually, the control seems to be more vital and in better condition. A comparison chart showing the mean cell density of the dermis is shown in Figure 8. Statistical significance was determined with a t-test between each condition. The control's thickness was less accurate indicating that there was high variance in the data. The P-value refers to the probability, assuming the null hypothesis of no effect or no difference, of obtaining an observed result equal to or more extreme than what was actually observed. Here, the letter "P" stands for probability and represents the likelihood that any observed variation between groups can be attributed to chance. The p-value between the "control" and the "no EGF" group is 0.008, and between the "no EGF" and the "human skin" is 0.001. The p-value between the "control" and the" human skin" is 0.5454. The p-values calculated in the "control" group is deemed in closer resemblance to the "human skin" group. Although using no EGF has lowered the variability in epidermal thickness throughout the organoid, more work needs to be done to make it consistent with human skin. Utilizing no EGF has a significant effect on what human skin organoids will look like and it indicates that the amount of EGF to use needs to be further studied.

Conclusion

Our analyses of organoids grown with and without EGF have led us to significant findings that challenge our initial hypothesis and therefore, show that our initial hypothesis is disproved. Contrary to our expectations, we have discovered that the absence of EGF in the growth medium hinders progress toward our primary objective of creating highly accurate human skin organoids. The density of living cells in the no EGF organoids is notably lower compared to the control group, indicating that the removal of EGF slows down cell proliferation and deviates further from the characteristics of real human skin. However, we observed that the average cornified thickness of the control organoids was 68.6 pixels (1.8 x 10^4 um), whereas it was 128.8 pixels (3.4 x 10^4 um) for the no EGF group. In comparison, human skin displayed an average cornified thickness of 47.4 pixels (1.2 x 10^4 um). Organoids grown without EGF exhibit a closer resemblance to human skin in terms of cornified thickness and the overall morphologies of the no-EGF organoids more closely resemble human skin compared to the control group, which can be attributed to the cornified-to-live thickness ratios. Furthermore, our statistical analysis yielded a significant p-value ($p<0.05$) between the no EGF and control groups, further supporting the observed distinctions. Despite both positive and negative findings, it is evident that the absence of EGF results in outcomes that lack certain aspects in one area while closely aligning with certain aspects in another area. This indicates the importance of striking a balance of EGF within the spectrum of no EGF and EGF to achieve the creation of an accurate human skin organoid.

Limitations

The organoids were cultured and grown over a two-week period. Growing them for longer can give more accurate results. Calculating density and thickness required counting the number of cells on the image taken by the microscope. However, this was done manually with the use of Supervisely. [34,35] There can be errors in how many cells were in the organoids, which, therefore, can alter the numbers slightly from our results.

Future Direction

No EGF and EGF seem to be extreme versions of human skin as they both have pros and cons with both. However, more work needs to be done to find a balance between the two. To confirm that no EGF is farther than getting to recreate human skin, a comparison of no EGF and control organoids can be furthered by grafting onto a mouse to test the viability and functionality of the two over time. Right now, it seems that EGF is a factor necessary in culturing organoids. The next steps are to experiment with different amounts of EGF and record the data to see what amount of EGF is necessary for human skin organoids. Machine learning programs could be utilized for faster and more accurate data collection. Deep learning-trained models for image analysis would allow for a larger data set with less human error. In this regard, the medical image toolbox from MATLAB would be quite applicable. MATLAB can also be used to collect the data and predict the fine line between how much EGF should be used to create an accurate skin organoid.