science, engineering and health studies

Research Article

Science, Engineering and Health Studies
https://lio1.tci-thaijo.org/index.php/sehs
ISSN (Online): 2630-0087

Effect of keratinocytes culture on the construction of fibrin-based human skin equivalents

Catalina Gaviria-Agudelo[1,2], Natalia Becerra[1], Luis Alfonso Correa[1,3,4], Sergio Estrada[1] and Luz Marina Restrepo[1,5]*

[1] Tissue Engineering and Cell Therapy Research Group, School of Medicine, University of Antioquia, Medellín 050010, Colombia
[2] European Research Institute for the Biology of Ageing, University Medical Center Groningen, Groningen 9713, The Netherlands
[3] Dermatology Department, School of Medicine, University of Antioquia, Medellín 050010, Colombia
[4] Laboratorio Clínico VID, Congregación Mariana, Medellín 050012, Colombia
[5] Medical Research Institute, School of Medicine, University of Antioquia, Medellín 050010, Colombia

***Corresponding author**:
Catalina Gaviria-Agudelo
catalina.gaviriaa@udea.edu.co*

***Received**: 27 July 2022*
***Revised**: 24 October 2022*
***Accepted**: 28 October 2022*
***Published**: 13 December 2022*

***Citation**:
Gaviria-Agudelo, C., Becerra, N., Correa, L. A., Estrada, S., and Restrepo, L. M. (2022). Effect of keratinocytes culture on the construction of fibrin-based human skin equivalents. Science, Engineering and Health Studies, 16, 22030008.*

ABSTRACT

Human skin equivalents are *in vitro* model constructed with human skin cells. The function and morphology of primary cells are particularly dependent on the cell isolation and culturing method, crucial in achieving reliable skin tissues. In this study, we show preliminary findings on the effect of the source of keratinocytes on the generation of 3D skin models. Two approaches were used to obtain keratinocytes: explant culture and a feeder layer of mouse fibroblasts. Skin samples were taken from three patients and processed by both methods. For the construction of HSEs, explant culture and feeder layer-derived keratinocytes were seeded on top of a fibroblasts-populated fibrin matrix. The histology and expression of epidermal markers were assessed by hematoxylin-eosin staining and immunohistochemistry, respectively. The integrity of the epidermal barrier was examined by measuring the transepithelial electrical resistance. To a greater or lesser extent, both methods produced HSEs where keratinocytes were able to stratify and express epidermal differentiation markers. The integrity of tight junctions (protein complexes that form the epidermal barrier) was enhanced in models composed of passage 1 cells. Feeder layer-derived keratinocytes generated HSEs with a healthier, thicker, and more properly stratified epidermis, improving the histological features, when compared to explant culture-derived models.

Keywords: explant culture; feeder layer; fibrin gel; human skin equivalents; transepithelial electrical resistance

1. INTRODUCTION

Human skin equivalents (HSEs) are skin substitutes created using keratinocytes, fibroblasts, and extracellular matrix products. In recent years, research on HSEs and subsequent development have progressed considerably, leading to outstanding epidermal and full thickness models. Those accepted by international bodies, such as the European Centre for the Validation of Alternative Methods, are composed of human keratinocytes seeded on special substrates. These cells are cultivated to develop a differentiated epidermis that resembles the *in vivo* structure and permits the application of chemical products (Vinardell and Mitjans, 2008; Zhang and Michniak-Kohn, 2012).

Cell isolation and culture are crucial steps in the production of HSEs. Today, the most common methods for

isolating and expanding keratinocytes from the skin are based on explant cultures and feeder layers. In the former, skin fragments are placed on cell culture dishes and air-dried until attachment is achieved. Keratinocytes migrate from the skin fragments occupying the culture's surface, and fibroblasts do not grow until some days later. This method is rapid, simple, and limits the potentially harmful effects of enzymatic treatments. However, fibroblasts may outgrow keratinocytes and the low complex media commonly used may limit the final epidermal cell yield (Orazizadeh et al., 2015). Also, calcium-containing media can result in keratinocytes differentiating rapidly, decreasing their proliferative capacity and applicability in future applications.

The second approach is based on the study of Rheinwald and Green (1975). Keratinocytes are disaggregated by proteolytic enzymes, cultured in a highly complex media, and seeded on lethally irradiated mouse fibroblasts that promote keratinocyte growth (Dragúňová et al., 2013; Gragnani et al., 2007). Among its many advantages, this protocol has led to high expansion rates (Green, 2008; Llames et al., 2004; Rheinwald and Green, 1975) and preservation of keratinocyte stemness and regenerative capacity. There has been no evidence of adverse effects on cell growth and development due to the use of mouse cells (Gray et al., 1983; Green, 2008).

Although the benefits of the co-culturing method have been widely described, investigations on keratinocyte growth focus mainly on monolayer cultures and little is known about the impact of cell expansion methods at the 3D level. In the present study, we compared the ability of explant and feeder layer-derived keratinocytes to produce 3D fibrin-based skin equivalents. To this end, morphological, immunohistochemistry, and transepithelial electrical resistance (TEER) analyses were performed on skin models. The results of different cell passages are also presented.

2. MATERIALS AND METHODS

2.1 Materials

Skin samples were collected from blepharoplasties leftovers following informed consent. Surgeries were performed at the IPS Universitaria at the University of Antioquia. The samples were taken from 3 patients, 2 men and 1 woman, aged between 46 and 53 years. All skin sample procurement procedures were approved by the Bioethical Committee of the Medical Research Institute at the University of Antioquia (approval no. 002, code F-017-00).

Fibroblasts and keratinocytes were isolated from the skin samples. The 3T3/J2 cell line (murine fibroblasts) was kindly provided by Dr. Alvaro Meana (Tissue Engineering Unit, Centro Comunitario de Sangre y Tejidos del Principado de Asturias) and used as a feeder layer for keratinocytes. Cells were seeded in T25 flasks (Falcon™, Glendale, AZ, USA) and fed with two culture media. Low complex media consisted of Dulbecco's modified. Eagle medium (DMEM) containing 10% fetal bovine serum (FBS) and 1% antibiotics (penicillin-streptomycin) (all from Lonza, Myersville, MD, USA). The more complex media consisted of specialized media for keratinocytes, hereinafter keratinocyte medium (KM). This was composed of DMEM/Ham's F-12 nutrient mixture (3:1) (Sigma, St. Louis, MO, USA) containing 10% FBS, 1% antibiotics, 1% L-glutamine (Lonza), 24 µg/mL adenine, 0.4 µg/mL hydrocortisone, 5 µg/mL insulin, 1.3 ng/mL triiodothyronine (all from Sigma), and 8 ng/mL cholera toxin (Life Technologies, Eugene, ORM USA). Epidermal growth factor (EGF, Austral Biologicals, San Ramon, CA, USA) was added at a later stage.

For epidermal immunostaining, the following antibodies were used: mouse monoclonal anti-cytokeratin 14, mouse monoclonal anti-loricrin, and mouse monoclonal anti-integrin (all from Abcam, Cambridge, MA, USA). Ethanol/xylene and Tris-buffered saline with Tween 20 (TBST) were kindly provided by the Pathology Department of The School of Medicine of the University of Antioquia.

For TEER measurements, a Millicell-ERS2 volt-ohm meter (Millipore, Bedford, MA, USA) was used.

Other reagents such as 0.25% trypsin/ethylenediaminetetraacetic acid (EDTA), phosphate buffered saline (PBS), mitomycin C, paraformaldehyde, and $CaCl_2$ were obtained from Sigma-Aldrich, while 70 µm cell strainers and 12-well plate 3460 Transwell inserts were purchased from Corning (Glendale, AZ, USA). NaCl and tranexamic acid were produced by Corpaul (Medellín, Colombia) and Ropsohn (Bogotá, Colombia), respectively.

2.2 Skin cells isolation and culture

Each skin sample was divided into 2 equal pieces, 1 cultured by the explant method and the other by using a feeder layer. For the former, the protocol described by Morales et al. (2016) was followed. Briefly, skin biopsies were fragmented and located on the surface of T25 culture flasks with no media to allow attachment. Next, low complex medium was added. Both keratinocytes and fibroblasts were obtained.

For the second approach, described by Rheinwald and Green (1975), 3T3/J2 cells were treated with mitomycin C and used as a feeder layer for keratinocytes. Skin samples were fragmented, stirred in trypsin/EDTA, and incubated under standard conditions. The suspension was then filtered through a cell strainer and disaggregated cells were seeded in T25 flasks containing 3T3/J2 cells and KM medium. After 3 days, EGF (10 ng/mL) was added to KM medium, henceforth referred to as KME. The medium was changed every 2-3 days. Fibroblasts were also obtained by seeding cell suspensions in T25 flasks containing low complex medium.

To investigate the effect of cell passage number, propagation of keratinocytes obtained by both methods was performed on mitomycin c-treated 3T3 fibroblasts, since explant-derived cells failed to grow in sufficient numbers on non-coated dishes. Thus, for each sample, 4 culture conditions were used: 1) passage 1 (P1) keratinocytes obtained by explant culture, 2) passage 2 or 3 (P>1) keratinocytes obtained by explant culture, 3) P1 keratinocytes obtained by feeder layer, and 4) P>1 keratinocytes obtained by feeder layer. These keratinocytes were used to generate HSEs.

2.3 HSEs construction

The protocol of Morales et al. (2016) was followed to generate the dermal compartment, mixing 217 µL human plasma, 1% tranexamic acid, 0.9% NaCl, and 100 µL of a fibroblasts solution (0.7×10^4 cells/mL). Next, 1% $CaCl_2$ was added to stimulate fibrin gel formation. The solution was carefully mixed, poured into 12-well plate inserts, and incubated for 15 min.

Once the dermal gel had formed, keratinocytes were harvested by using 0.25% trypsin/EDTA. For co-cultures, 3T3/J2 cells were first detached with 0.05% trypsin/EDTA and the remaining keratinocytes were then obtained following 0.25% of trypsin/EDTA treatment. $3x10^4$ keratinocytes were placed on top and the bi-layered structure was cultured in KM for 3 days and then in KME. On day 7, the culture was exposed to the air-liquid interface and cultured for over 14 days to promote keratinocyte differentiation. For every culture condition, at least 3 HSEs were produced.

2.4 Histology and immunohistochemistry

After 21 days in culture, HSEs were fixed in 10% paraformaldehyde, dehydrated in alcohol, and embedded in paraffin blocks. Hematoxylin-eosin (H&E) staining was performed in 5-µm tissue sections.

For immunostaining, 4-µm tissue sections were washed and rehydrated with ethanol/xylene. Primary antibodies were incubated for 1 h at room temperature and, after 2 washes with TBST, horseradish peroxidase was used for the subsequent color-based reaction.

2.5 TEER measurement

TEER measurement was used to evaluate the integrity of tight junctions as an indicator of the tightness of the HSEs epidermal barrier (Srinivasan et al., 2015). On day 21, the culture media was removed and 800 µL of PBS were added to both the apical and basolateral sides. A pair of electrodes were immersed into the insert and the outer well.

To measure impedance, a squared wave current was applied. Results from at least three different measurements for each tissue replicate were recorded. The mean values are expressed in ohms per unit of surface area.

2.6 Statistical analysis

Graph plots and statistical analysis were performed in Graphpad Prism 8 software and are shown as mean ± standard error of mean (SEM). The number of experimental replicates and statistical significance (p-value) are indicated in the figure legends. For analysis, a two-tailed t-test was applied. $^{*}p<0.05$; $^{**}p<0.01$, $^{****}p<0.0001$, and n.s. represents "no significant difference".

3. RESULTS

3.1 Layer-derived HSEs show a higher histological resemblance to native skin

On day 21, HSEs were evaluated histologically by H&E staining (Figure 1). The dermal and epidermal compartments can be clearly distinguished in all skin models. Feeder layer-derived HSEs at P1 (Figures 1a and 1b) displayed a thick and healthy epidermis with columnar basal cells. Four different epidermal strata were also formed: basal stratum (blackhead arrow), spinous stratum (bluehead arrow), granular stratum (redhead arrow), and corneum stratum (greenhead arrow), and a dermis with well-distributed fibroblasts was observed.

Despite displaying a thick epidermis, keratinocytes from explant-derived HSEs seemed to be unorganized and the four layers are not easily distinguishable (Figures 1d-1f). Unlike samples 1 and 2, HSEs derived from sample 3 showed impairment in the differentiation process, regardless of the cell culturing method (Figures 1c and 1f) and a certain degree of parakeratosis (Figures 1c, 1f, 1l). This demonstrated the heterogeneity in HSEs produced by keratinocytes from different donors.

On the other hand, P>1 keratinocytes formed a tissue where the epidermis was not as continuous and thick as expected, and the formation of the four epidermal differentiation stages is not always present (Figures 1g-1l). In some cases, there was no evidence of stratum corneum formation (Figures 1g, 1h, 1j, and 1k), and basal cells were already flattened (Figures 1i-1l).

Together, these results indicated that keratinocytes grown on a murine 3T3 feeder layer were able to produce skin models with more morphological similarities to native skin, especially in early passages.

3.2 Feeder layer-derived keratinocytes produce HSEs with more epidermal cell layers

Next, the epidermal thickness of the skin models was assessed by counting the number of cell layers in the epidermis of different H&E image sections (Figure 2a). Significant differences were found between different culturing methods and between HSEs composed of P1 and P>1 keratinocytes. Feeder layer-derived HSEs provided significantly more epidermal layers when compared to the corresponding explant culture-derived models. P1 keratinocytes of both explant culture and feeder layer, gave rise to tissues with a higher number of cell layers, doubling the corresponding P>1 counterpart (compare blue bars with orange ones).

These data show that 1) feeder layer-derived keratinocytes give rise to HSEs with greater epidermal thickness, and 2) higher keratinocyte passage numbers can significantly impair the final epidermal thickness, regardless of the culturing method. As continuously dividing undifferentiated keratinocytes generates all the epidermal strata, these results are suggestive of basal state preservation during feeder layer culture and an association between keratinocyte differentiation and passage number.

3.3 Effect of cell passage on TEER values

As TEER values are indicative of the strength of the tight junctions, the integrity of the barrier function of HSEs was evaluated by measuring the TEER after 21 days of culture (Figure 2b). There were no differences in TEER between cell culture approaches, while significant differences were found between HSEs composed of P1 and P>1 keratinocytes. P1 keratinocytes of both explant culture and feeder layer gave rise to tissues with tighter epidermal barriers when compared to the P>1 counterpart.

These data suggest that P>1 keratinocytes may show synthesis and/or functional defects of tight junction proteins when producing HSEs. Differences in the stratum corneum formation as well as the number of cell layers can also affect the integrity of the epidermal barrier.

3.4 Effect of source of keratinocyte on expression of epidermal markers

As keratinocytes express specific proteins along their differentiation path, we examined the presence of cytokeratin 14 (K14), integrin, and loricrin in HSEs (Figure 3). K14 is one of the major structural proteins of stratifying epithelia and is synthesized by the basal proliferative layer (Rugg et al., 1994). Although it is present in all layers, both explant culture and feeder layer-derived HSEs showed strong K14 expression (Figures 3a, 3d, 3g, and 3j), at comparable levels to that of human skin (Figure 3m).

Integrin α6β4 is one of the components of hemidesmosomes and serves as an anchoring site between the dermis and epidermis (Geuijen and Sonnenberg. 2002). Integrin levels were higher in the basal stratum of all skin models (Figures 3b, 3e, 3h, and 3k), although to a lesser extent than in native skin (Figure 3n). Loricrin (the major component of the cornified envelope) expression was examined to evaluate the formation of the horny layer. Even if faint, loricrin was found in the horny layer of both the feeder layer and explant culture HSEs (Figures e 3c, 3f, 3i, and 3l). Its presence in P>1 derived HSEs in which incomplete differentiation was found (Figures 1g-1l), was indicative of premature differentiation (Figure 3f and 3l).

These findings indicated that cytokeratin 14, integrin, and loricrin expression in HSEs were not considerably affected by the keratinocyte culturing method or passage number.

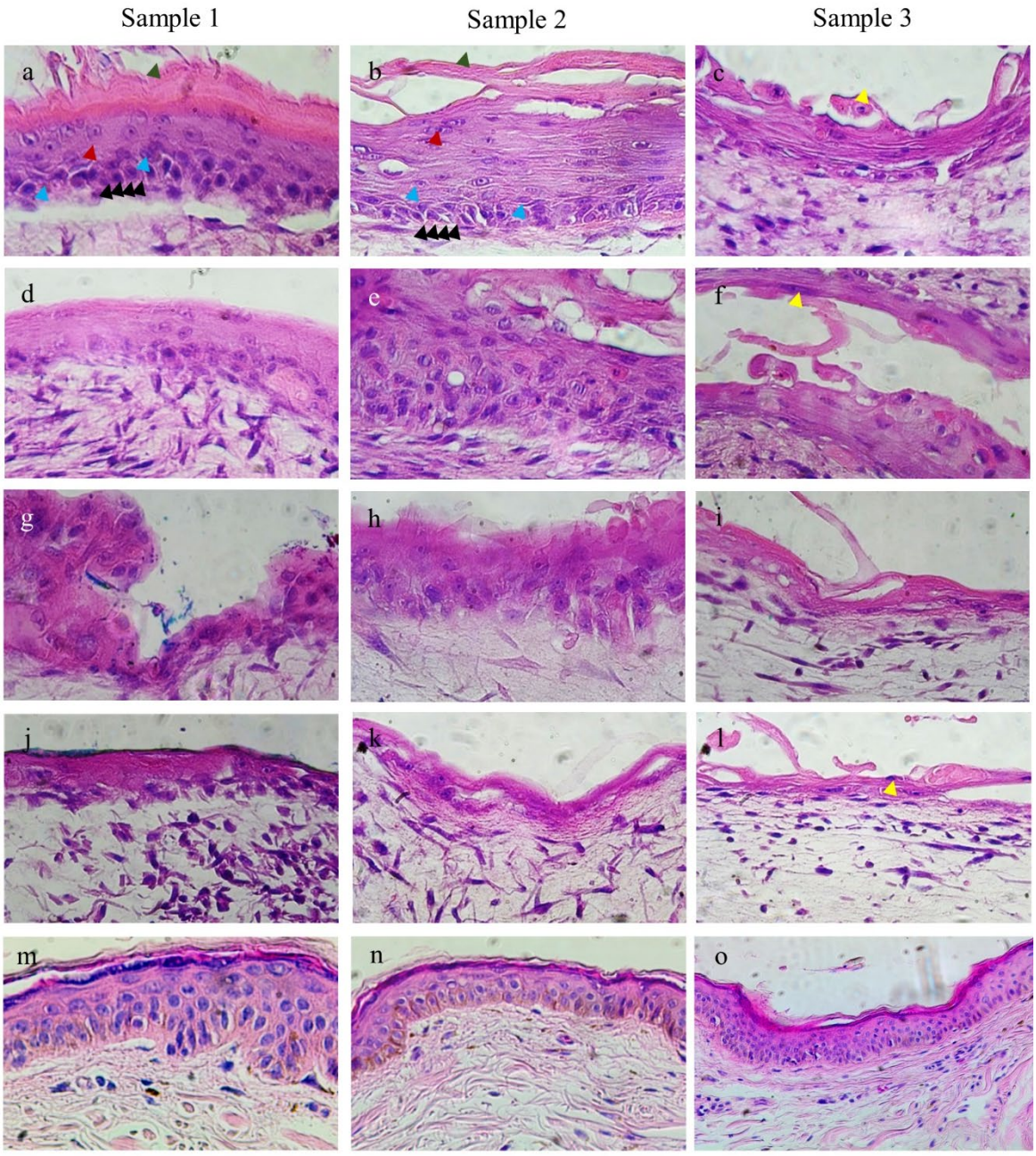

Figure 1. Histological characteristics of human skin equivalents (HSEs) determined by hematoxylin-eosin staining: (a), (b) and (c) P1 feeder layer-derived HSEs of samples 1, 2, and 3 at 80x, respectively; (d), (e) and (f) P1 explant culture-derived HSEs of samples 1, 2, and 3 at 80x, respectively; (g), (h) and (i) P>1 feeder layer-derived HSEs of samples 1, 2 and 3 at 80x, respectively; (j), (k) and (l) P>1 explant culture-derived HSEs of patients 1, 2 and 3 at 80x, respectively. (m), (n), and (o) native skin at 80X, 40X, and 20X magnification, respectively

Note: Blackhead arrow: basal layer, bluehead arrow: spinous layer, redhead arrow: granular layer, greenhead arrow: horny layer, and yellow head arrows: parakeratosis.

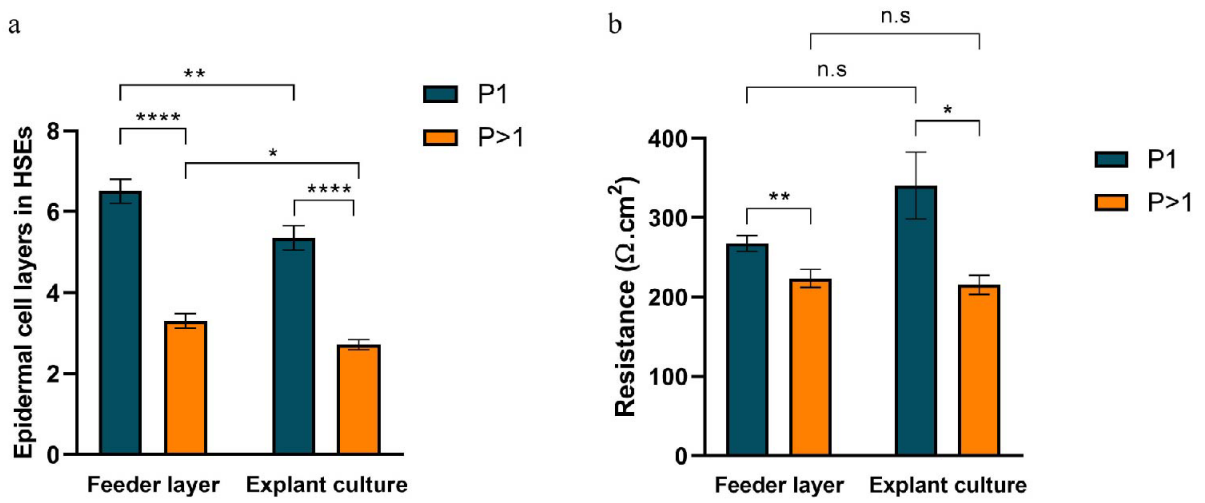

Figure 2. Characterization of human skin equivalents composed of cells obtained by explant culture or feeder layer in terms of (a) number of epidermal cell layers, (b) transepithelial electrical resistance
Note: Data represent the mean ± SEM of at least 7 tissues per culture condition. A two-tailed t-test was applied for statistical analysis, n.s: non-significant; $^{*}p \leq 0.05$; $^{**}p \leq 0.01$, $^{****}p \leq 0.0001$.

4. DISCUSSION

This study compared the ability of feeder layer-derived and explant culture-derived keratinocytes to generate 3D fibrin-based skin models. The feeder layer-derived keratinocytes was found to produce 3D models with improved morphological characteristics, compared to explant culture-derived HSEs. The results further suggested that barrier function defects could arise as cell passage increased.

We first confirmed that the yield of keratinocytes following co-culture with murine fibroblasts was significantly higher than that of the explant method (data not shown). Feeder layers are known to release extracellular matrix proteins and growth factors that promote keratinocyte growth (Dragúňová et al., 2013; Gragnani et al., 2007), can suppress keratinocytes differentiation, detoxify the culture medium, act as a substrate for cell attachment, and extend keratinocytes' life span (Gray et al., 1983; Green, 2008). These results were therefore, indicative of proliferative and undifferentiated state preservation at the 2D level, which, in this study, was hypothesized to be advantageous for 3D epidermal formation.

For explants, the final yield depended on the number of skin fragments, the presence of a proliferative-promoting agent (i.e., growth factors or 3T3 cells), and calcium concentration in culture media. Several investigations have highlighted the need to use low calcium levels during the first days of culture as this reduces the proliferation rate and promotes sequential differentiation (Bikle et al., 2012; Borowiec et al., 2013; Elsholz et al., 2014; Pillai et al., 1990). Calcium-containing media along with the absence of a proliferative promoting agent likely contributed to the lower number of keratinocytes obtained by explants.

Orazizadeh et al. (2015) compared the enzymatic and explant methods for monolayer cultures of keratinocytes. In contrast to these results, they found no attachment of skin cells to either the feeder layer or type I collagen scaffold, perhaps, because the seeding cell density may have been low or the trypsin treatment harsh. However, the authors found that explant keratinocytes could grow for multiple passages and expressed pan-cytokeratin when plated on a collagen scaffold surface, confirming the importance of having extracellular matrix support for the long-term growth of keratinocytes.

In terms of 3D models, histology results showed that P1 feeder layer-derived keratinocytes generated tissues that more closely resembled the human epidermal structure. In native skin, each epidermal layer is characterized by a unique morphology and function of cells that differentiate while acquiring a more flattened appearance. This was particularly true of the P1 feeder layer-derived HSEs, which presented columnar-shaped cells in the basal layer and anucleated flat cells in the stratum corneum. In contrast, explant culture cells-derived HSEs presented a less organized differentiation process.

As for epidermal barrier analysis, P1 cells-derived HSEs showed significantly higher TEER when compared to P>1 cells-derived models, suggesting that the integrity of the barrier function improved when using "younger" cells and that cell health played an important role in epidermal development. In fact, the lowest TEER values corresponded to HSEs with the thinner and unstratified epidermis, as judged by H&E results. Although no statistical differences were found between cell culturing methods, explant culture-derived HSEs seemed to display higher TEER levels. Given that the number of epidermal layers is lower and stratum corneum formation is not improved in these tissues, the difference, if one exists, could be attributed to variations in the production and distribution of tight junction proteins. This should be confirmed in future studies. Considering that culturing time and the presence of calcium contribute to keratinocytes differentiation (Bikle et al., 2012; Borowiec et al., 2013; Elsholz et al., 2014; Pillai et al., 1990), early passage cultures were expected to contain higher numbers of cells with high proliferative capacity, which resulted in the formation of more cell layers, continuous basal and corneum strata, and stronger tight junctions.

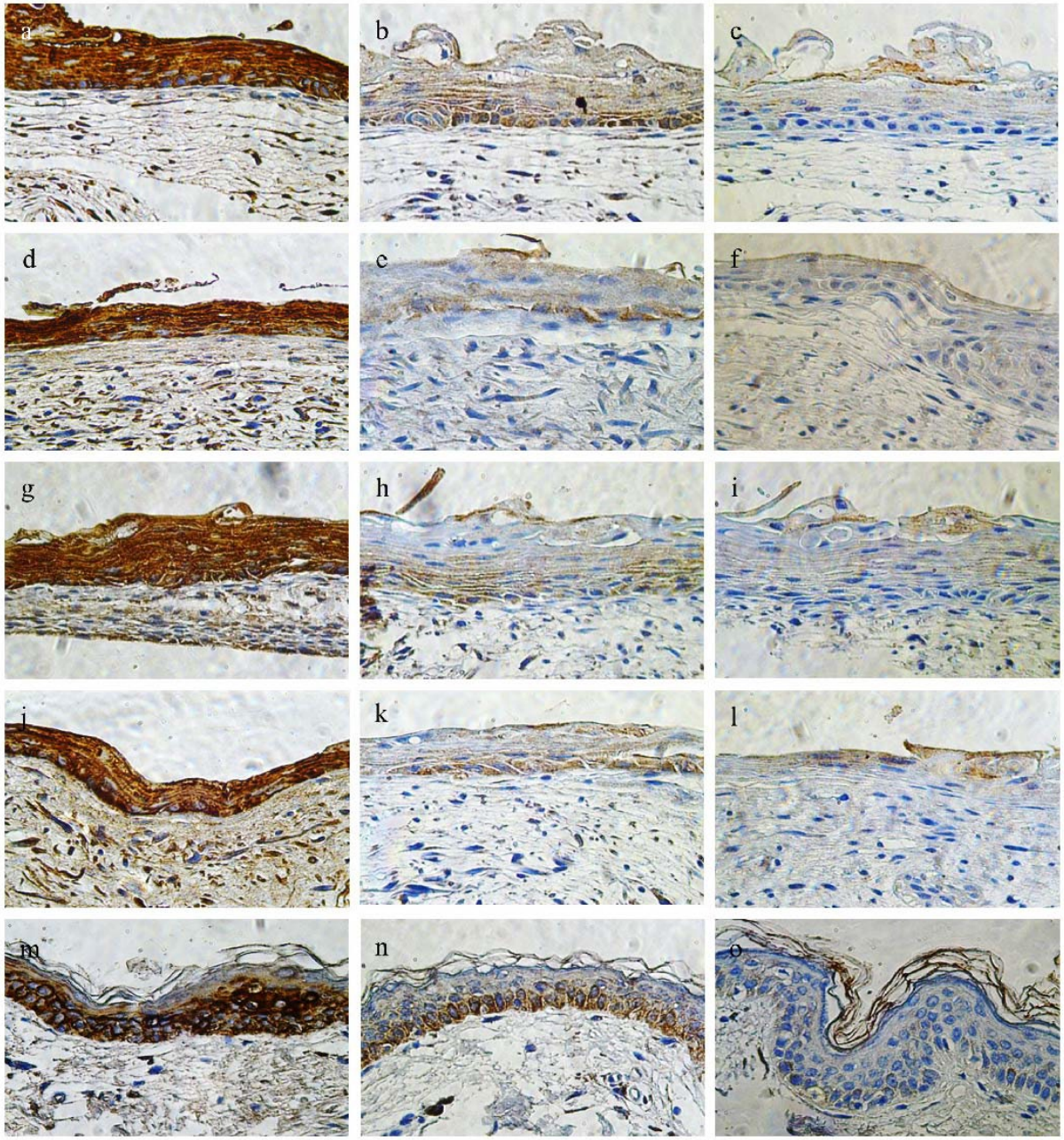

Figure 3. Expression pattern of human skin equivalents (HSEs) epidermal markers at 80x magnification: cytokeratin 14 expression of (a) P1 feeder layer HSEs, (d) P>1 feeder layer HSEs, (g) P1 explant culture HSEs, (j) P>1 explant culture HSEs, and (m) native skin; integrin expression of (b) P1 feeder layer HSEs, (e) P>1 feeder layer HSEs, (h) P1 explant culture HSEs, (k) P>1 explant culture HSEs, and (n) native skin; loricrin expression of (c) P1 feeder layer HSEs, (f) P>1 feeder layer HSEs, (i) P1 explant culture HSEs, (l) P>1 explant culture HSEs, and (o) native skin.

As keratinocytes expressed specific proteins along their differentiation path, the presence of cytokeratin 14, integrin, and loricrin in HSEs was examined. There were no considerable differences in the expression of differentiation markers between HSEs, regardless of the culturing method and passage number. All HSEs were positive for cytokeratin 14, integrin, and loricrin, revealing a stratification process. The presence of cytokeratin 14 in the suprabasal layers was indicative of the presence of basal cells in the upper layers, which should be verified in future experiments. The slightly stronger staining of integrin in P1 feeder layer-derived HSEs could be explained by the higher number of columnar-shaped cells, which were in charge of attaching to the basement membrane. Importantly, P>1 HSEs expressed loricrin despite presenting incomplete stratification, suggesting disturbances in the differentiation process. In fact, models lacking stratum corneum presented flattened basal cells in the first layers of the epidermis.

In skin research, keratinocytes are often used up to passage 3 with no decline in proliferation. Here, calcium-containing media was used to favor fibroblast survival and

growth. Hence, keratinocytes differentiation occurred in early passages, highlighting the negative effect of generating HSEs with differentiated cells. Given this study's sample size and access to old donor skin only, the association between keratinocyte proliferation capacity and epidermal development should be confirmed with more samples, as should the way in which the state of keratinocyte differentiation affects the expression of tight junction proteins and other epidermal markers.

These findings are especially useful in the pharmacological field, for which proper morphology and functional epidermal barrier should be verified before applying any chemical testing protocols (OECD, 2016; OECD, 2015). Knowing the level of keratinocyte differentiation in advance would facilitate the prediction of epidermal stratification and barrier generation. This will prevent the unnecessary fabrication of HSEs that will not meet the requirements.

5. CONCLUSION

This study showed promising results and highlighted the importance of employing undifferentiated and highly proliferative keratinocytes when HSEs are constructed. P1 feeder layer-derived cells are associated with improved fibrin-based skin models, emphasizing the importance of cell fitness when HSEs are constructed. We strongly encourage future research into skin equivalents to focus more on the stemness and proliferative potential of keratinocytes. The characterization of keratinocyte differentiation state, proliferative rate, and the association of these factors with the development of a reliable epidermis could be of great help when constructing HSEs.

REFERENCES

Bikle, D. D., Xie, Z., and Tu, C. L. (2012). Calcium regulation of keratinocyte differentiation. *Expert Review of Endocrinology & Metabolism*, 7(4), 461-472.

Borowiec, A. S., Delcourt, P., Dewailly, E., and Bidaux, G. (2013). Optimal differentiation of *in vitro* keratinocytes requires multifactorial external control. *PLOS One*, 8(10), e77507.

Dragúňová, J., Kabát, P., and Koller. J. (2013). Skin explant cultures as a source of keratinocytes for cultivation. *Cell and Tissue Banking*, 14, 317-324.

Elsholz, F., Harteneck, C., Muller, W., and Friedland, K. (2014). Calcium - a central regulator of keratinocyte differentiation in health and disease. *European Journal of Dermatology*, 24(6), 650-661.

Geuijen, C. A. W., and Sonnenberg, A. (2002). Dynamics of the alpha6beta4 integrin in keratinocytes. *Molecular Biology of the Cell*, 13(11), 3845-3858.

Gragnani, A., Sobral, C. S., and Ferreira, L. M. (2007). Thermolysin in human cultured keratinocyte isolation. *Brazilian Journal of Biology*, 67(1), 105-109.

Gray, T. E., Thomassen, D. G., Mass, M. J., and Barrett. J. C. (1983). Quantitation of cell proliferation, colony formation, and carcinogen induced cytotoxicity of rat tracheal epithelial cells grown in culture on 3T3 feeder layers. *In Vitro*, 19(7), 559-570.

Green, H. (2008). The birth of therapy with cultured cells. *BioEssays*, 30(9), 897-903.

Llames, S. G., Del Rio, M., Larcher, F., García, E., García, M., José Escamez, M., Jorcano, J. L., Holguín, P., and Meana, A. (2004). Human plasma as a dermal scaffold for the generation of a completely autologous bioengineered skin. *Transplantation*, 77(3), 350-355.

Morales, M., Pérez, D., Correa, L., and Restrepo, L. (2016). Evaluation of fibrin-based dermal-epidermal organotypic cultures for *in vitro* skin corrosion and irritation testing of chemicals according to OECD TG 431 and 439. *Toxicology in Vitro*, 36, 89-96.

OECD. (2016). *Test No. 431: In Vitro Skin Corrosion: Reconstructed Human Epidermis (RHE) Test Method*. André Pascal: OECD Publishing, pp. 1-26.

OECD. (2015). *Test No. 439: In Vitro Skin Irritation: Reconstructed Human Epidermis Test Method*. André Pascal: OECD Publishing, pp. 1-28.

Orazizadeh, M., Hashemitabar, M., Bahramzadeh, S., Dehbashi, F. N., and Saremy, S. (2015). Comparison of the enzymatic and explant methods for the culture of keratinocytes isolated from human foreskin. *Biomedical Reports*, 3(3), 304-308.

Pillai, S., Bikle, D. D., Mancianti, M. L., Cline, P., and Hincenbergs, M. (1990). Calcium regulation of growth and differentiation of normal human keratinocytes: modulation of differentiation competence by stages of growth and extracellular calcium. *Journal of Cellular Physiology*, 143(2), 294-302.

Rheinwald, J. G., and Green, H. (1975). Serial cultivation of strains of human epidermal keratinocytes: The formation keratinizing colonies from single cells is. *Cell*, 6(3), 331-343.

Rugg, E. L., McLean, W. H., Lane, E. B., Pitera, R., McMillan, J. R., Dopping-Hepenstal, P. J., Navsaria, H. A., Leigh, I. M., and Eady, R. A. (1994). A functional knockout of human keratin 14. *Genes & Development*, 8(21), 2563-2573.

Srinivasan, B., Kolli, A. R., Esch, M. B., Abaci, H. E., Shuler, M. L., and Hickman, J. J. (2015). TEER measurement techniques for *in vitro* barrier model systems. *SLAS Technology*, 20(2), 107-126.

Vinardell, M. P., and Mitjans, M. (2008). Alternative methods for eye and skin irritation tests: An overview. *Journal of Pharmaceutical Sciences*, 97(1), 46-59.

Zhang, Z., and Michniak-Kohn, B. B. (2012). Tissue engineered human skin equivalents. *Pharmaceutics*, 4(1), 26-41.