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A Reliable Procedure to Cultivate Three-dimensional Reconstructed Human Epidermis at a Large Scale

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TITLE:

Cultivating a Three-dimensional Reconstructed Human Epidermis at a Large Scale

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KEYWORDS:

Reconstituted human epidermis, human epidermal equivalents, primary keratinocytes, epidermis, in vitro studies.

SUMMARY:

This protocol describes a straightforward method to cultivate three-dimensional reconstituted human epidermis in a reproducible and robust manner. Additionally, it characterizes the structure-function relationship of the epidermal barrier model. The biological responses of the reconstituted human epidermis upon proinflammatory stimuli are also presented.

ABSTRACT:

A three-dimensional reconstituted human epidermis model is presented that is reconstructed from neonatal primary keratinocytes. Herein, a protocol for the cultivation process and the characterization of the model is described. Neonatal primary keratinocytes are grown submerged on permeable polycarbonate inserts and lifted to the air-liquid interface three days after seeding. After fourteen days of stimulation with defined growth factors and ascorbic acid in high calcium culture medium, the model is fully differentiated. Histological analysis revealed a completely stratified epidermis, mimicking the morphology of native human skin. To characterize the model and its barrier functions, protein levels and localization specific for early-stage keratinocyte differentiation (i.e., keratin 10), late-stage differentiation (i.e., involucrin, loricrin, and filaggrin) and tissue adhesion (i.e., desmoglein 1), were assessed by immunofluorescence. The tissue barrier integrity was further evaluated by measuring transepithelial electrical resistance. Reconstituted human epidermis was responsive to proinflammatory stimuli (i.e., lipopolysaccharide and tumor necrosis factor alpha), leading to increased cytokine release (i.e., interleukin 1 alpha and interleukin 8). This protocol represents a straightforward and

reproducible in vitro method to cultivate reconstituted human epidermis as a tool to assess environmental effects and a broad range of skin-related studies.

INTRODUCTION:

The epidermis is the outermost layer of the skin, at the direct interface between the human body and the external environment. Its main functions are to provide protection and hydration[1]. The epidermis acts as an effective physical barrier against external agents and prevents excessive water loss from the body. These skin functions mainly depend on the cellular arrangement in the outermost layers of the skin, the composition, and organization of intercellular lipids[2]. The epidermis is primarily composed of keratinocytes that migrate upwards to the outer side of the tissue and undergo differentiation. There are 4-5 epidermal layers that are characterized by their stage of differentiation. From the inside to the outside, the epidermal layers start from the viable epidermis, i.e., the stratum basale (SB), the stratum spinosum (SS), and the stratum granulosum (SG), to the non-viable uppermost layer, i.e., the stratum corneum (SC)[3]. The basal layer is mainly composed of proliferating keratin-enriched keratinocytes, which start differentiating to migrate through the SS[4]. During the keratinocyte maturation, various changes in protein expression and structure occur. Keratinocytes adhere through the formation of desmosomal junctions[5]. In the SG, the generation of lamellar bodies is initiated. They consists of lipid precursors and enzymes that are crucial for the formation of the skin barrier function[6]. The SG is also characterized by the presence of keratohyalin granules in the cytoplasm of the keratinocytes. At the interface with the stratum corneum, the contents of the lamellar bodies are extruded into the intercellular spaces and the non-polar lipids such as ceramides, cholesterol, and free fatty acids organize into stacked lamellar lipid bilayers to form the extracellular lipid matrix[7]. In the SC, cells lose all cellular organelles including the nucleus, due to enzymatic degradation processes and adopt a flattened morphology. They become surrounded by a cornified envelope made of cross-linked protein layers, and are referred to as corneocytes[8, 9]. Desmosomal components are cross-linked to the cornified envelope to form corneodesmosomes and bind the corneocytes together. The resulting epithelium is continually renewed from stem cells, with a turnover time of approximately 5-6 weeks[10]. The differentiation process of the keratinocytes, which results in a fully stratified epidermis, is crucial for the formation of the barrier function of the skin[11].

During wounding and inflammation, keratinocytes induce changes in adhesion molecules and surface receptors and trigger proinflammatory responses via secretion of cytokines, chemokines, and antimicrobial peptides[12]. The skin is not only a physical barrier against exogenous substances; it also acts as an immune sensor upon exposure to pathogens. In addition, it regulates the diffusion of several substances across its layers, such as water content to protect the human body from dehydration. The skin is also involved in the synthesis of vitamin D and has various other metabolic functions[3, 13, 14].

To assess the adverse effects of exogenous substances, toxicologists have relied for decades on animal testing, but nowadays it is not the preferred approach. Besides having limited predictive capacity for human toxicity, animal models involve numerous ethical issues. The ban on animal testing in the cosmetic industry and the recommendation to follow the 3R principle (i.e., Replacement, Reduction, and Refinement) in research have led to the development of alternative test methods based on in vitro approaches[15]. The first in vitro skin cell models have already been

described in the 90's, and an impressive development from simple human keratinocyte mono-cultures to fully differentiated epidermis and full-thickness barriers has been achieved[16]. Nowadays, skin tissue engineering has gained importance in both the pharmaceutical and dermato-cosmetic fields. In the last two decades, several companies have commercialized three-dimensional (3D) reconstructed human epidermis (RhE) that represent standardized and reproducible tools for skin-related studies. Several commercial RhE models are accepted for in vitro skin testing of chemicals according to OECD guidelines for the testing of skin irritation[17, 18] (i.e., test guideline 439[19]) and skin corrosion[20] (i.e., test guideline 431[21]). The in vitro test for skin sensitization[22] (i.e., SENS-IS assay) is currently in the approval track and under peer-review[23]. There are also numerous other assays developed that utilize commercial RhE models, to evaluate phototoxicity[24], to test drug formulations[25], cosmetic formulations and active ingredients[26], to study the skin barrier function[27] and to test the biological response to environmental stressors[28–31].

In addition to commercially available 3D skin models, multiple research groups have developed their own RhEs[32–37]. In-house RhEs offer the advantage for controlling the culture conditions according to the purpose of the study. Specifically, researchers can select the type and the source of the keratinocytes to be used for the reconstitution of their 3D epidermal model (i.e., primary vs. immortalized, neonatal vs. aged, single vs. pooled random donors, sex, ethnicity, individual living habits such as smoking, etc.). They have the possibility to vary the composition of the culture medium and incorporate growth factors, vitamins, or other compounds that can modulate the expression of target proteins or lipids. With in-house RhEs, researchers can also investigate biological responses and biomechanical properties as a function of the differentiation state of the 3D model. In addition to those intuitive parameters, there are continuous efforts to increase the complexity of 3D skin models and make them more physiologically relevant, for instance by adding other epidermal cell types (e.g. melanocytes and immune cells)[38, 39], by culturing the keratinocytes on top of a fibroblast-populated collagen matrix[40–42], and by including components of the vascular network[43–45].

Although it is possible to tune the culture conditions according to specific needs, there are parameters that must be respected to guarantee both the quality and relevance of a 3D RhE. To cultivate RhE tissues, normal human epidermal keratinocytes (NHEKs) are seeded into specific permeable culture inserts whose porous synthetic membrane separates the wells into two compartments, i.e., the apical and basolateral compartment. The porosity of the membrane (i.e., a pore size of 0.4 µm) is such that it allows the formation of a cell monolayer in the apical compartment with no migration of cells to the basal insert side, and the feeding of the keratinocytes with essential nutrients from the culture medium contained in the basolateral compartment. At the beginning of the reconstitution process, NHEKs are cultured in submerged conditions for a few days to allow their adhesion with the membrane. The calcium level in both compartments is increased compared to the calcium concentration used for the 2D culture of NHEKs to slow down the proliferation of cells and promote instead their differentiation[46]. An epidermal calcium gradient is essential to regulate the barrier formation and homeostasis[47, 48]. High-calcium levels (i.e., up to 1.5 mM) promote the formation of intercellular junctions and modulate the formation of the cornified envelope during terminal differentiation[49]. Once

keratinocytes form a continuous and tightly adherent monolayer on the supporting membrane, the medium from the apical compartment is removed and culture conditions continue at the air-liquid interface (ALI) to stimulate stratification and establish an epidermal barrier[50, 51]. Specific culture conditions are crucial to obtain a fully stratified epithelium[36]. During the reconstitution process at ALI, the medium in the basolateral compartment is supplemented with keratinocyte growth factor (KGF), insulin, calcium, and ascorbic acid. Ascorbic acid plays a major role in the formation of an appropriate SC lipid barrier, closely resembling the human native skin[52]. Keratinocytes grown in ascorbic acid-supplemented medium demonstrate a differentiated phenotype, with an enhanced number of keratohyalin granules, as well as organized intercellular lipid lamellae in the interstices of the corneocytes[52]. Such supplementation is essential to improve epidermal barrier function by increasing cornified envelope content and avoiding depletion of hydrophilic antioxidant stores[53, 54]. KGF, an important paracrine mediator of epidermal proliferation and differentiation, is used to stimulate the NHEKs[55].

The main downsides of in-house RhEs include the loss of standardization between research institutions and increased labor intensity and time consumption (up to 3 weeks compared to the ready-to-use commercial models). The aim of the present paper is to address these drawbacks, setting the basis for production at a larger scale. In addition to the abovementioned advantages of in-house RhEs, the current protocol aims to reduce the intra- and inter-variability among tissues, to reduce contamination risks, and to streamline the cultivation process.

The current protocol describes a reproducible and robust method to cultivate RhEs using neonatal NHEKs. Moreover, it shows representative results of the characterization of the RhEs morphology, barrier integrity, and expression of proteins that are specific for epidermal differentiation. RhEs morphological structure was examined using hematoxylin and eosin (H&E) staining and transmission electron microscopy (TEM). To evaluate the barrier integrity, the trans-epidermal electrical resistance (TEER) and the exposure time to Triton X-100 to reduce 50% of the tissue viability (ET_{50}) was measured. The formation of desmosomal junctions (i.e., desmoglein 1) was analyzed by immunofluorescence (IF) to evaluate keratinocyte adhesion. The formation of epidermal structural proteins (i.e., involucrin, loricrin, and filaggrin) was evaluated and detected with IF. These proteins are involved in the formation of the highly cross-linked protein envelope that surrounds SC corneocytes and as a result are important markers for late-stage epidermal differentiation[56, 57]. Additionally, IF was used to analyze keratin 10, a protein induced in early-stage differentiated cells in the stratum spinosum[58] and found inside all differentiated layers. Finally, the RhE's response to proinflammatory stimuli (i.e., lipopolysaccharide and tumor necrosis factor alpha) was investigated. The levels of interleukin 1 alpha (IL-1α) and interleukin 8 (IL-8) were measured in the cell culture media, using enzyme-linked immunosorbent assays (ELISA).

PROTOCOL:

Review and adhere to national and international ethical considerations and conditions related to the use of human tissues or cells before planning and executing any research activity involving this protocol.

NOTE: All steps of this protocol must be carried out in aseptic conditions. Biosafety Level 2 practices are the minimum requirement for the cultivation of RhEs. All necessary safety precautions must be taken when handling the chemicals/reagents described in this protocol.

1. Preparation of cell culture media

NOTE: There are three different types of serum-free culture media used for the cultivation of RhEs **(Table 1)**: (i) the *basal medium* with low calcium level (60 µM Ca^{2+}) used for the 2D culture of NHEKs; (ii) the *submerged medium* with high calcium level (1.5 mM Ca^{2+}) used for the seeding of NHEKs into the cell culture insert system; and (iii) the *air-liquid interface medium* with high calcium level (1.5 mM Ca^{2+}), ascorbic acid, and KGF.

[Place **Table 1** Here]

1.1. Prepare the basal medium.

1.1.1. Supplement the bottle of 500 mL of keratinocyte growth medium (**Table of Materials**) with 5 mL of human keratinocyte growth supplements (HKGS) in order to reach final concentrations of 0.2% [v/v] bovine pituitary extract (BPE), 0.2 ng/mL human recombinant epidermal growth factor (EGF), 0.18 µg/mL hydrocortisone, 5 µg/mL bovine transferrin, and 0.01 µg/mL of recombinant human insulin-like growth factor-I (IGF-I).

1.1.2. Add 5 mL of 100x antibiotic-antimycotic.

1.2. Prepare the submerged medium.

1.2.1. Supplement the bottle of 500 mL of keratinocyte growth medium (**Table of Materials**) with 5 mL of HKGS in order to reach final concentrations of 0.2% [v/v] BPE, 0.2 ng/mL human recombinant EGF, 0.18 µg/mL hydrocortisone, 5 µg/mL bovine transferrin, and 0.01 µg/mL of human recombinant IGF-I.

1.2.2. Add 5 mL of 100x antibiotic-antimycotic.

1.2.3. Add 5 mL of a 0.144 M $CaCl_2$ stock solution to reach a final concentration of 1.5 mM Ca^{2+}.

NOTE: The calcium concentration is already increased during the submerged phase to stimulate the differentiation of the keratinocytes and initiate the stratification process[49].

1.3. Prepare the air-liquid interface (ALI) medium.

1.3.1. Supplement one bottle of 500 mL of keratinocyte growth medium (**Table of Materials**) with 5 mL of HKGS in order to reach final concentrations of 0.2% [v/v] BPE, 0.2 ng/mL human

recombinant EGF, 0.18 µg/mL hydrocortisone, 5 µg/mL bovine transferrin, and 0.01 µg/mL of human recombinant IGF-I.

1.3.2. Add 5 mL of 100x antibiotic-antimycotic.

1.3.3. Add 5 mL of a 0.144 M $CaCl_2$ stock solution to reach a final concentration of 1.5 mM Ca^{2+}.

1.3.4. Add 1 mL of a 25 mg/mL ascorbic acid stock solution to reach a final concentration of 50 µg/mL ascorbic acid.

1.3.5. Add 50 µL of a 100 µg/mL KGF in 1% [w/v] bovine serum albumin in PBS stock solution to reach a final concentration of 10 ng/mL KGF.

NOTE: Since ascorbic acid is sensitive to oxidation, it is recommended to use a more stable ascorbic acid-derivate, such as magnesium l-ascorbyl-2-phosphate[59] or L-ascorbic acid 2-phosphate sesquimagnesium[60]. If ascorbic acid is used, it is recommended to freshly supplement the ALI medium with ascorbic acid before each refresh.

2. Culture of NHEKs

NOTE: Since primary human keratinocytes remain proliferative upon their fourth or fifth passage[61], NHEKs in their third passage are used for the cultivation of RhEs. Primary keratinocytes should be handled very carefully due to their high sensitivity. Careful and slow pipetting of cell suspensions at any time is very important, to not disturb the condition of the cells.

2.1. Thaw a vial with 1 x 10^6 cryopreserved NHEKs in a water bath at 37 °C, by submerging part of the vial in the water. Incubate the vial for 1-2 minutes in the water bath, until only a small sliver of ice is visible.

CAUTION: Do not submerge the whole vial in the water bath to avoid contaminations. Do not thaw the cells longer than 2 minutes; this can reduce the cell viability. Wipe the vial with a 70% ethanol solution before transferring the tube into the laminar hood.

2.2. Resuspend the cells very carefully, by pipetting up and down 2-3 times. Transfer the cell suspension into two T75 flasks containing a total of 15 mL of pre-warmed thawing medium, resulting in a seeding density of 6.7 x 10^4 cells/cm^2.

NOTE: For the first two passages and the thawing of cryopreserved NHEKs, cell culture medium is used according to the supplier's recommendations.

2.3. Place the flasks into the cell culture incubator (37 °C, 5% CO_2, and 95% relative humidity (RH)).

2.4. After approximately 24 hours, replace the thawing medium by the basal medium to remove dimethyl sulfoxide (DMSO) from the keratinocyte freezing solution.

2.5. Refresh the basal medium every two days.

2.6. After 4-6 days of cultivation, the cells should be around 80% confluent and ready for seeding in inserts for the cultivation of RhEs.

NOTE: Keratinocytes should be grown to maximum 80% confluence to preserve their proliferative capacity[62]. The number of cells to be thawed must take into consideration several parameters, such as the cell passage number, the cell viability upon thawing, the seeding efficiency as well as the doubling time.

3. Seeding of NHEKs

NOTE: This protocol is designed for use within a 24-well plate format. If other plate formats are required (e.g., 12-well or 6-well format), optimization to adapt for the correct seeding density is necessary. **Figure 1** summarizes a proposed timeline for RhE cultivation and shows the cultivation conditions.

[Insert **Figure 1** here]

3.1. Pre-fill 24-well plates with 1.5 mL of submerged medium, ideally using a dispenser pipette.

3.2. Remove the basal medium from the T75 flasks used for the culture of the NHEKs.

3.3. Rinse the cells by adding 5 mL of pre-warmed phosphate-buffered saline (PBS) to each T75 flask.

3.4. Remove PBS from the flasks.

NOTE: This step is crucial, since the medium contains proteins and calcium that will inhibit the trypsin activity.

3.5. Add 2-3 mL of pre-warmed 0.05% [v/v] trypsin/ethylene diamine tetra acetic acid (EDTA) to each T75 flask. Make sure that the trypsin solution is equally distributed on the cell culture area of the flask.

CAUTION: The 2 mL volume is based on the 80% confluence mentioned above. Use 3 mL for flasks with a higher confluency.

3.6. Place the flasks for 4 minutes in the cell culture incubator (37 °C, 5% CO_2, and 95% RH). Check whether the cells detach using the microscope at a 10x magnification. Rap the flask to help the

cells release from the surface of the flask. Detached cells can be observed as rounded cells floating in the trypsin solution.

CAUTION: Do not incubate the cells in trypsin for longer than 6 minutes. Over-trypsinization can damage the cells and decrease their adherence[63].

3.7. Once all the cells are detached, add an equal volume (i.e., 2-3 mL) of pre-warmed trypsin inhibitor to each T75 flask.

3.8. Transfer the cell suspension from the flasks to a centrifuge tube.

3.9. Rinse the flasks with 5 mL of pre-warmed PBS and transfer it to the centrifuge tube containing the cell suspension.

NOTE: Make sure that most of the cells are collected by checking the number of residual cells in the flasks under the microscope. The surface of the flask should be 95% empty. If this is not the case it is possible to repeat the trypsinization step 3.3-3.9. Note however that re-trypsinization should be avoided.

3.10. Centrifuge the harvested cells at 400 *x g* for 5 min.

3.11. Carefully discard most of the supernatant, leaving approximately 100-200 µL in the tube.

CAUTION: Do not aspirate the pellet during this procedure. Gently flick the tube with your fingers to carefully dissolve a part of the cell pellet in the supernatant.

3.12. Gently resuspend the pellet of cells in a low volume of submerged medium, pipette up and down 5-10 times to ensure a uniform cell suspension. Start with a low volume (i.e., 500 µL) to avoid the formation of cell aggregates and add up to 1 mL of submerged medium in total per initial T75 flask.

NOTE: Gently flick the tube with fingers to carefully dissolve a part of the cell pellet in the supernatant.

3.13. Count the cells in the suspension using the trypan blue exclusion method.

3.13.1. Dilute 0.4% [v/v] trypan blue stain and the cell suspension in a 1:1 ratio, by adding 10 µL of 0.4% [v/v] trypan blue stain to 10 µL of cell suspension. Add 10 µL of the solution to a counting slide. Measure the cell count immediately after mixing the cell suspension with trypan blue, since trypan blue starts to decrease cell viability after exposure longer than 1 min[65].

CAUTION: Trypan blue was shown to be a potential mutagen, carcinogen, and teratogen[64]. Handle the dye with care and dispose of the waste safely according to local laboratory regulations.

NOTE: An alternative approach to the use of trypan blue is the non-hazardous dye Erythrosin B[66].

3.14. Dilute the cell suspension with additional submerged medium to reach a concentration of 3.525 x 10^5 cells/mL in submerged medium by adding the volume V_2 as shown in equation 1:

$$V_2 = C_1 * \frac{V_1}{C_2} - V_1 \qquad (1)$$

C_1 = counted cell concentration in the cell suspension obtained in 3.12 (cells/mL)
V_1 = volume used to resuspend the pellet of cells in 3.12 (mL)
C_2 = targeted cell concentration in the suspension (i.e., 3.53 x 10^5 cells/mL)
V_2 = volume to be added to reach the targeted cell concentration (mL)

NOTE: The surface area of the recommended culture insert is 0.47 cm^2; therefore, the corresponding seeding density is 3.75 x 10^5 cells/cm^2.

3.15. Perform a second cell count (C_3) of the diluted solution obtained in step 3.14. Use equation 2 to calculate the cell suspension volume (V_4) to be seeded into the culture insert:

$$V_4 = C_3 * \frac{V_3}{C_4} \qquad (2)$$

C_3 = targeted cell concentration in the suspension (i.e., 3.53 x 10^5 cells/mL)
V_3 = targeted volume of the cell suspension to be seeded in the culture insert (i.e., 0.5 mL)
C_4 = counted cell concentration in the diluted suspension obtained in 3.14 (cells/mL)
V_4 = actual volume of the cell suspension to be seeded in the culture insert (mL)

3.16. Hang the 24 cell culture inserts in the highest position of the carrier plate and transfer the carrier plate to the 24-well plate pre-filled with submerged medium (cf. 3.1).

CAUTION: When transferring the carrier plate to the multi-well plate, ensure that no air bubbles are trapped between the insert membrane and the submerged medium from the basal compartment, as this will affect the feeding of the cells and ultimately compromise the RhE viability and morphology.

3.17. Add the determined volume V_4 (from equation 2) of the cell suspension to each insert.

NOTE: It is preferred to use the reverse pipetting technique to avoid the formation of air bubbles when dispensing the cell suspension to the culture inserts.

CAUTION: Make sure not to damage the membrane when dispensing the cell suspension into the culture insert. A precaution is to dispense the cell suspension along the wall of the insert system without touching the surface of the membrane.

3.18. After seeding, incubate the 24-well plates for 10-15 min at room temperature, to overcome an edge effect (i.e., non-uniform temperature distribution between all wells[67]). Do not move the plates during this time.

3.19. Transfer the plates to the cell culture incubator (37 °C, 5% CO_2, and 95% RH). The cells remain in submerged conditions for three days.

NOTE: To avoid tissue variability, do not stack the plates in the incubator after seeding to make sure that each insert receives the same amount of heat. After three days (i.e., after ALI), stacking of plates is possible.

4. Cultivation at ALI

4.1. After a three-day incubation in the cell culture incubator (37 °C, 5% CO_2, and 95% RH), expose the cells that have adhered to the membrane surface to the ALI by removal of the submerged medium from the apical compartment using an aspiration system and a glass Pasteur pipette.

NOTE: Alternatively, the submerged medium from the apical compartment can be removed with a manual micropipette.

4.2. Fill new 24-well plates with 1.5 mL of fresh pre-warmed ALI medium and transfer the carrier plates with the culture inserts to the new multi-well plates.

4.3. Transfer the multi-well plates back to the cell culture incubator (37 °C, 5% CO_2, and 95% RH).

4.4. Refresh the ALI medium every 2-3 days for 14 days.

4.5. Perform the refresh in two steps: 1) prepare a new plate containing 1.5 mL/well of fresh pre-warmed ALI medium and 2) transfer the carrier plate to the new plate.

CAUTION: During the entire reconstitution procedure it is best not remove the lid covering the carrier plate to keep the RhEs protected from potential contamination.

NOTE: The ALI step is crucial for the development of a stratified epidermal model as it allows terminal differentiation of the keratinocytes[68]. After going to ALI, a visual control of the inserts is required, to check whether there are 'leaky tissues': medium droplets on the tissue surface coming for the basolateral compartment. If the leakage happens at ALI day 3, remove the medium from the tissue surface. If the leakages persist, it is recommended to discard those tissues as it is an indication that there is no correct barrier formation in the RhE model.

4.6. For RhE harvesting, collect the inserts and cell culture medium for histological analysis, viability assays, protein/RNA extraction, and ELISAs.

REPRESENTATIVE RESULTS:

NHEKs in 2D culture display a traditional morphology with a consistent polygonal shape (**Figure 2A**). As described above, NHEKs are seeded into culture inserts after reaching a confluency of approximately 80%. The morphology of the RhEs was analyzed using hematoxylin and eosin (H&E) staining and TEM. After 15 days at ALI, the RhEs form a fully stratified tissue as indicated by its four main epidermal layers: the SB, the SS, the SG, and the SC (**Figure 2B**). In the SB layer, the cells have a columnar shape. From the second layer on towards the upper layers of the RhE, the cells start to change their shape. NHEKs differentiate from having a columnar shape in the SB layer, towards a spinous shape in the SS layer. In the SG layer, the cells have a more flattened shape and display keratohyalin granules (KG) that are represented as purple dots in the cytoplasm. Their characteristic round and stellar shape are highlighted by white arrows on the H&E image (**Figure 2C**). The cells in the SC, are terminally differentiated and are completely flattened and lack a cell nucleus. The stratified RhEs have an overall thickness of 84.3 ± 2.4 µm and their stratum corneum has a thickness of 19.6 ± 3.2 µm (**Figure 2D**). These values are comparable to those reported for native human skin, i.e., 60-120 µm and 10-20 µm, respectively[69]. The number of viable layers is 6-7, which is lower compared to that of native human skin, being approximately 7-14[70]. Ultrastructural analysis of RhEs at different time points in the reconstitution protocol (i.e., 7, 10, 13, and 15 days) reveals the cornification process of the RhEs with an increased number of corneocyte layers over time (**Figure 2E**). After 15 days at the ALI, the SC of the RhE tissue is made of approximately 15-25 layers, which is comparable to the value reported for native human skin (i.e., 15-20 layers)[69].

[Insert **Figure 2** here]

NHEKs in RhEs show different protein expression profiles according to their differentiation stage. The expression of proteins specific for early-stage keratinocyte differentiation (i.e., keratin 10), late-stage keratinocyte differentiation (i.e., involucrin, loricrin, and filaggrin), and keratinocyte adhesion (i.e., desmoglein 1) in RhEs was determined using immunofluorescence (IF) staining. Involucrin expression appears more predominantly located in the SG layer since its expression is initiated earlier during the differentiation process (**Figure 3D**), whereas the expression of filaggrin and loricrin is located in the upper layers (**Figure 3B-C**). Keratin 10 expression was found in all the viable layers, except of the SB layer (**Figure 3E**). RhEs display functional desmosomal junctions, as indicated by the expression of desmoglein 1 in the intercellular space of the viable epidermal layers (**Figure 3F**). To conclude, all five markers are expressed and located in the appropriate epidermal layers and translate to a healthy epidermal differentiation process.

[Insert **Figure 3** here]

The barrier properties of the RhE model was investigated by assessing both the tissue viability upon topical treatment with a known barrier disruptor and the tissue integrity. The tissue integrity was determined after 15 days by measuring the TEER using a voltohmmeter. The 2567 ± 415 $\Omega.cm^2$ values recorded for the RhEs translate the formation of a continuous barrier (**Figure 4A**). Those values are in range with those reported for reconstituted human epidermal models[71–74]. Additionally, the required exposure time for a cytotoxic reference chemical (i.e., Triton X-100)

to reduce the tissue viability by 50% (ET_{50}) was determined with a thiazolyl blue tetrazolium bromide (MTT) assay. The ET_{50} value measured for the RhE was 2.1 hours. This value falls within the acceptance range of other 3D epidermal models that are qualified for reliable prediction of irritation classification (OECD Guideline 439)[19].

[Insert **Figure 4** here]

Responsiveness of RhEs was investigated upon known proinflammatory stimuli. RhEs were treated systemically, i.e., addition of stimuli in medium of basolateral compartment, using 100 µg/mL lipopolysaccharide (LPS) and 40 ng/mL tumor necrosis factor alpha (TNF-α). After 24 hours of stimuli, the cell culture medium was harvested. The cytotoxicity was measured using a lactate dehydrogenase (LDH) assay and compared to values of a known membrane disruptor, the Triton X-100 detergent (**Figure 5**). A significant increase ($p > 0.05$, one-way ANOVA, Dunnett's multiple comparison test) was shown in LDH release in RhEs treated with Triton X-100. LPS and TNF-α treatments both showed not be cytotoxic.

[Insert **Figure 5** here]

The release of interleukin 1 alpha (IL-1α) and interleukin 8 (IL-8) in the RhE medium was quantified using enzyme-linked immunosorbent assays (ELISAs). **Figure 6** shows both the quantified and relative IL-1α and IL-8 release by the RhEs upon challenge with LPS and TNF-α. LPS treatment resulted in a statistically significant ($p > 0.05$, unpaired Student's T-test) upregulation in the release of IL-8 (9.6 ± 1 fold increase) and IL-1α (2.7 ± 1.3 fold increase). TNF-α did not upregulate IL-8 (2.3 ± 0.8 fold increase), but resulted in a statistically significant ($p > 0.05$, unpaired Student's T-test) upregulation of IL-1α (1.8 ± 0.5 fold increase).

[Insert **Figure 6** here]

Figure 1: Schematic timeline of the protocol. Presentation of the 3D RhE model preparation, cultivation process, and application (exposure to chemical substance). The scheme includes the appropriate cell culture media types for each step.

Figure 2: Primary keratinocytes and reconstituted human epidermis. (**A**) Phase-contrast microscopy image of primary keratinocytes before seeding onto inserts. Scale bar is 50 µm. (**B-C**) H&E bright field microscopy image of RhE. Scale bar is 50 µm (**B**) and 25 µm (**C**). (**D**) Quantification of thickness of RhE and stratum corneum (mean ± SEM, n=3). (**E**) Transmission electron microscopy images of RhE after 7, 10, 13, and 15 days at air-liquid interface. Scale bar is 4 µm.

Figure 3: Epidermal differentiation, tissue adhesion, and tissue integrity of reconstituted human epidermis. (**A**) H&E bright field microscopy image of RhE. Confocal fluorescence microscopy images of (**B**) filaggrin (FLG), (**C**) loricrin (LOR), (**D**) involucrin (INV), (**E**) keratin 10 (K10), and **(F)** desmoglein 1 (DSG-1) represented in magenta. Nuclei staining (DAPI) is represented in blue. Scale bar is 25 µm.

Figure 4: Barrier properties of reconstituted human epidermis. (**A**) Tissue integrity measured with trans-epithelial electrical resistance (mean ± SEM, n=6). (**B**) ET_{50} determined by measuring tissue viability (i.e., MTT assay) upon topical exposure to 78.3 µL of 1% Triton X-100 (mean ± SEM, n=3).

Figure 5: The cytotoxicity measured via lactate dehydrogenase (LDH) assay. The data is presented as relative values to CTRL; mean ± SEM, n=9 (TX-100), n=8 (LPS), n=3 (TNF-α). Significance was tested with one-way ANOVA, Dunnett's multiple comparison test. Asterisk denotes statistically significant increase compared to CTRL, ****p < 0.0001).

Figure 6: Proinflammatory reactions in the reconstructed human epidermis. The concentration of IL-8 release of the RhE upon a 24-hour challenge with LPS (**A**) and TNF-α (**B**)**.** Data is represented as mean ± SEM, n=8 (LPS), n=3 (TNF-α). (**C**) Data is represented as the mean of relative value compared to CTRL ± SEM, n=8 (LPS), n=3 (TNF-α). The concentration of IL-1α release of the RhE upon a 24-hour challenge with LPS (**D**) and TNF-α (**E**)**.** Data is represented as mean ± SEM, n=8 (LPS), n=3 (TNF-α). (**F**) Data is represented as the mean of relative value compared to CTRL ± SEM, n=4 (LPS), n=3 (TNF-α). Significance was tested by an unpaired Student's T-test. Asterisk denotes statistically significant decrease compared to CTRL, *p < 0.05, ****p < 0.0001).

Table 1. Summary table of the different culture media used to cultivate RhEs. List of different culture media with supplements.

DISCUSSION:

RhEs are widely used as screening tools in the pharmaceutical and dermato-cosmetic fields[36, 75–78]. Although several companies have made such RhEs commercially available, they remain costly and limit the possibility to vary cultivation parameters as required for new research questions. This paper describes the production procedure of in-house RhEs in a robust and reliable manner and provides a detailed characterization of the obtained tissues to confirm the relevance of the model as an alternative approach to animal testing.

Some of the steps in the protocol are crucial to assure correct RhE differentiation and reproducibility. This can be carried out by utilizing the optimal cells, medium type(s), and cultivation conditions. In the proposed RhE model, neonatal NHEKs were selected for their lack of antigenic exposure, compared to adult NHEKs. Furthermore, keratinocytes were limited to Caucasian ethnicity to avoid inter-species variability. Primary keratinocytes are typically used for their ability to differentiate and stratify[79]. They can be obtained commercially or by in-house isolation from adult skin[80]. The cultivation of a cell line (i.e., HaCaT) on a polycarbonate membrane, has shown to fail differentiation and demonstrated an impaired capacity to synthesize lipids that are necessary for barrier formation[81]. However, the inclusion of different culture matrices, such as hydrogels, collagen, fibrin, and spheroids cultures, resulted in the successful development of 3D skin models[78, 82–86]. The immortalized cell lines, N/TERT, have been shown to be suitable for the development of RhEs[35]. Primary keratinocytes remain proliferative upon their fourth or fifth passage[61], therefore the current protocol uses keratinocytes in their third passage. De Vuyst et al. have demonstrated that the cell seeding density is of importance

and needs to be sufficient (i.e., ≥ $2.5x10^5$ cells/cm^2) to ensure that the medium from the basolateral compartment does not diffuse to the apical compartment. An insufficient seeding density (i.e., < $2.5x10^5$ cells/cm^2) can result in an inability to form a proper barrier, which is indicated by the diffusion of medium from the basolateral to the apical compartment, resulting in submerged culture conditions instead of ALI[62]. Serum-free keratinocyte growth media (see **Table of Materials**) was preferred for reproducibility purposes, since it offers the advantage of working with a chemically defined medium and reduces the risk of contamination. This medium has a lower calcium concentration (i.e., 60 µM) and therefore stimulates the proliferation of keratinocytes[87]. Increasing the calcium concentration (i.e., 1.5 mM) from the first step of the RhE cultivation, favors the keratinocyte differentiation and skin barrier formation and homeostasis[49]. In addition, the ALI medium is supplemented with ascorbic acid, which has shown to be crucial for the formation of stratum corneum lipids and to promote differentiation[52–54]. The ALI medium also contains keratinocyte growth factor (KGF), which is a growth factor secreted by fibroblasts that can bind to keratinocyte transmembrane receptors and upon activation has a dual role in differentiation and wound repair[55, 88]. It is important to refresh the ALI medium at specific time intervals, to provide a constant supply of fresh nutrients to the RhEs. The use of a carrier plate system is crucial for RhE cultivation at a larger scale (i.e., 24 inserts/plate). It offers the advantage of saving time, reducing the risk of contamination, and leaves less room for the introduction of human errors. It also provides the possibility to culture RhEs in a high volume of media (i.e., 1.5 mL), which reduces the required number of ALI medium refreshes. Additionally, it offers the possibility to transfer a full plate of inserts to a plate with fresh medium, avoiding contact with the inserts individually or uncovering the plate lid.

There are several limitations of the RhE model that should be noted. In native human skin there is an equilibrium between the proliferation of keratinocytes in the basal layer and the detachment of corneocytes in the stratum corneum (i.e., desquamation)[89]. However, in vitro, desquamation does not take place. Therefore, the corneocytes remain attached to the RhE and form a thick stratum corneum that is less physiologically relevant. Hence, there is a limited cultivation timespan of RhEs. Moreover, this RhE model is simple and straightforward, since it consists of a singular cell type, i.e., the keratinocyte, which is the most abundant cell type of the epidermis. However, there are other cell types resident in the epidermis, such as melanocytes, dendritic cells (i.e., Langerhans cells), T cells (e.g., $CD8^+$ cells), and Merkel cells[13]. To enhance the physiological relevance of the skin model, researchers have made skin models more complex by adding melanocytes[38] , immune cells[39], or patient-derived cells[90]. One should keep in mind that the barrier properties of human skin models are different compared to native human skin, due to notably a different SC lipid composition and a higher barrier permeability [91–95]. However, several studies have reported on changes in barrier properties of human skin models by the cultivation under hypoxia[96] or decreased relative humidity[97], the modulation of the dermal matrix with Chitosan[98], and alteration in the free fatty acids in the culture medium[99]. Moreover, in both simple and more complex RhEs, the culture conditions and medium composition can be modulated to mimic pathological features. By challenging the model with cytokines, an abnormal morphology[100] and alterations in gene and protein expression levels can be established that are typically observed in common skin disorders, such as atopic dermatitis and psoriasis[35, 101–105]. Silencing specific genes in NHEKs prior to seeding of the RhEs, is another approach used to mimic

features of skin disorders and investigate new therapeutic solutions[106, 107]. Besides modeling an epidermal layer only, a dermal compartment can be included in the model (i.e., named human skin equivalents or full-thickness models) by embedding fibroblasts in a collagen matrix prior to RhE reconstruction, making it more physiologically relevant and suitable for aging and wound healing related studies[108–110]. Additionally, tumor spheroids have been added to human skin equivalents to study melanoma progression[111, 112]. The latest advances in the field of skin models are bio-printing and skin-on-a-chip. Multiple research groups have succeeded recently in the development of (perfusable) bio-printed skin equivalents[45, 113, 114]. The proposed protocol takes advantage of a 24-well format and a carrier plate, avoiding inserts to be handled individually. However, the study scale is still quite limited and lacks automation. By implementing the use of bio-printing or skin-on-a-chip, smaller skin models can be used with more automated processes and on a larger scale.

The RhE described in this protocol has multiple similarities to the already developed and well characterized commercial epidermal models. The morphological analysis has demonstrated that although the number of viable layers in the proposed RhE model is lower compared to that of native human skin (i.e., 6-7 compared to 7-14), it is comparable to that of the EpiDerm RhE model (i.e, 8-12)[70]. Similarly to the EpiDerm, EpiSkin, and SkinEthic models, the upper RhE layer shows a basket-weave pattern of densely packed corneocyte layers[28]. Moreover, TEM analysis revealed that the number of stratum corneum layers in the proposed RhE model (i.e., 15-25) is comparable to that of EpiDerm (i.e., 16-25)[70]. Overall, the proposed RhE model demonstrates a similar architecture to that of other commercialized epidermal models, mimicking the native human epidermis. The tissue integrity as measured by TEER is in range with commercial RhE models (i.e., between 3000-5600 $\Omega*cm^2$)[71–73] and other in-house RhEs (i.e., approximately 5000 $\Omega*cm^2$)[74, 115]. The proposed RhE model shows to be well differentiated, as indicated by the correct localization of differentiation and tissue adhesion markers. Moreover, the proposed RhE model shows to be responsive to proinflammatory stimuli (i.e., LPS and TNF-α).

To conclude, the current protocol shows how to produce RhEs in a reliable manner and at a relatively large scale to meet the needs of researchers in both academic and private institutions. The proposed RhE model shows to have similar morphology, epidermal differentiation, and biological responsiveness to other existing commercial models. It provides an alternative tool for both the pharmaceutical and dermato-cosmetic field when access to a relevant skin model is required.

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Marc Eeman and Benedetta Petracca are employees of Dow Silicones Belgium. All other authors have nothing to disclose.

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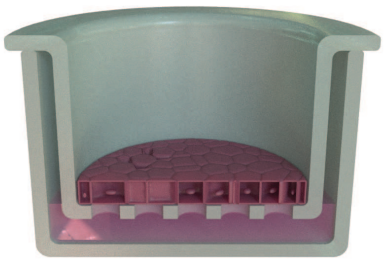
Figure 1

1. seed 1.8 x 10^5 cells in 500 µL/insert

Day -3

submerged medium
keratinocyte growth medium
+ 1.5 mM $CaCl_2$

2. remove medium from apical compartment



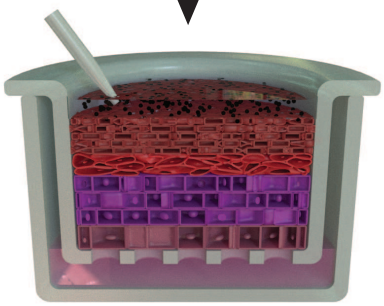
Day 0

air-liquid interface medium
keratinocyte growth medium
+ 1.5 mM $CaCl_2$
+ 50 µg/mL vitamin C
+ 10 ng/mL KGF

3. refresh medium every 2-3 days to stimulate diffferentiation

Day 2/Day 4/Day7/Day 9/Day 11

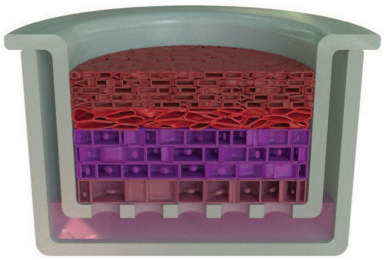
4. exposure of reconstituted human epidermis



Day 14

submerged medium
keratinocyte growth medium
+ 1.5 mM $CaCl_2$

5. harvest



Day 15/Day 16

Figure 2

Click here to access/download;Figure;Figure 2.jpg

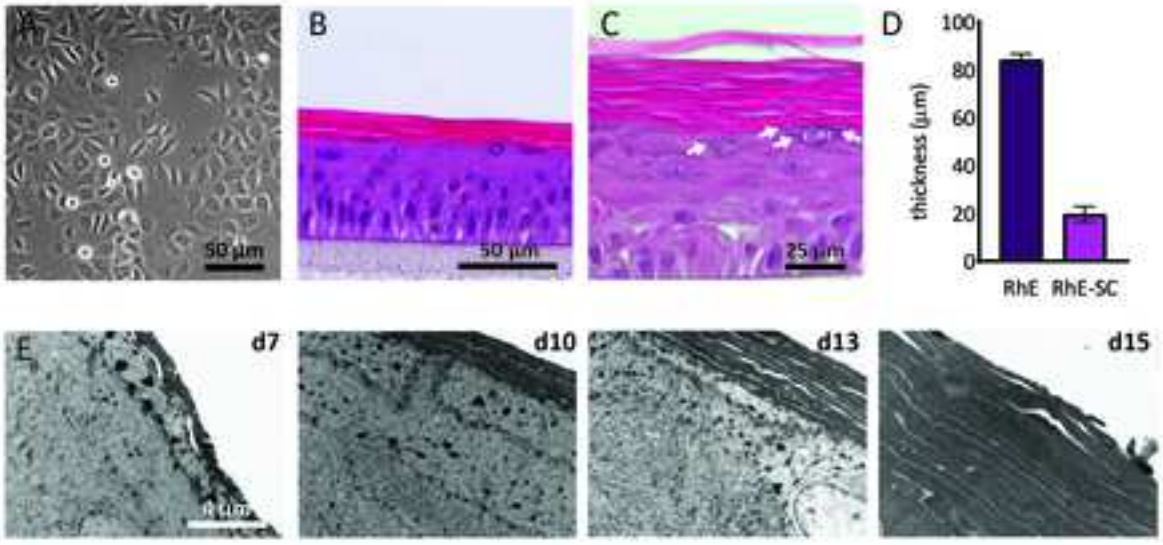
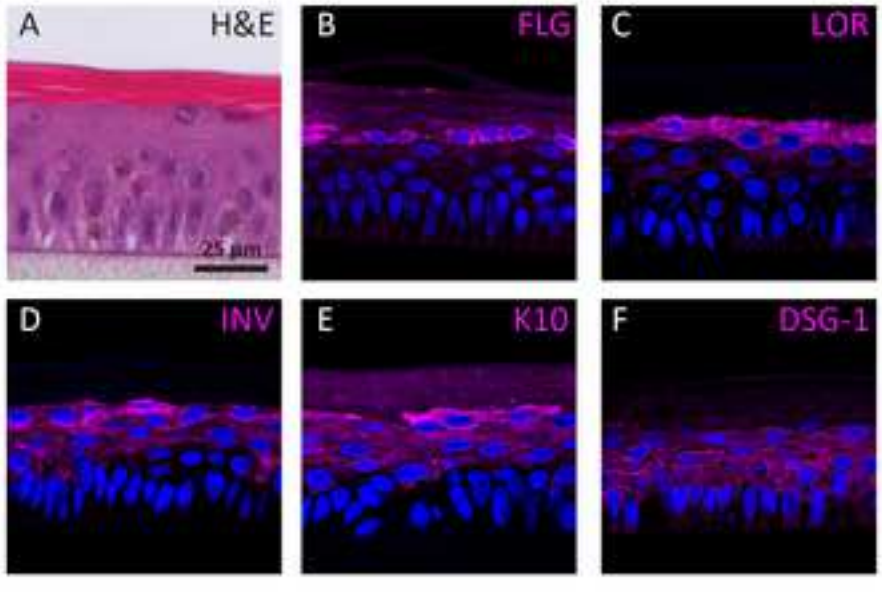


Figure 3

Click here to access/download;Figure;Figure 3.jpg



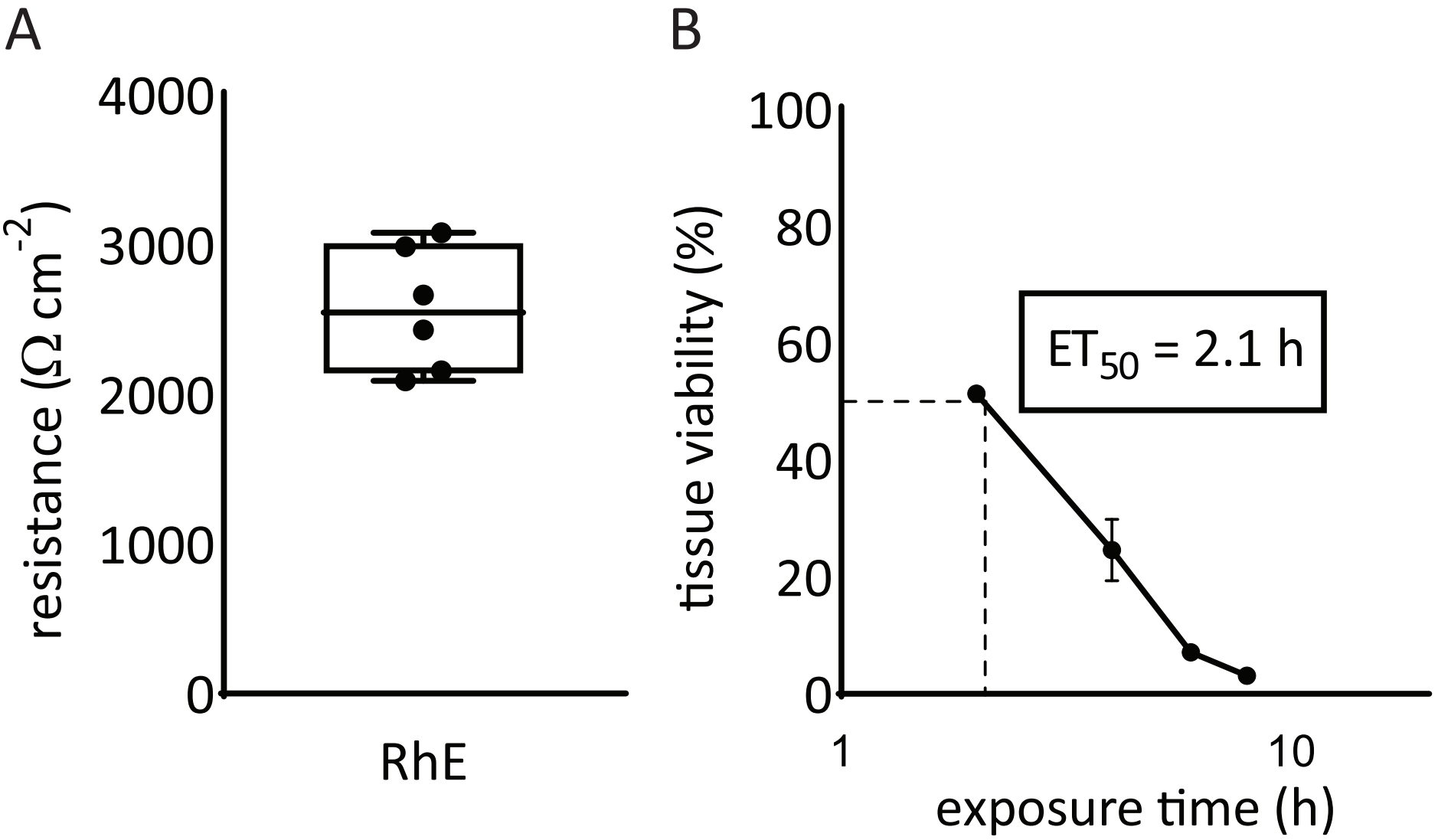


Figure 5

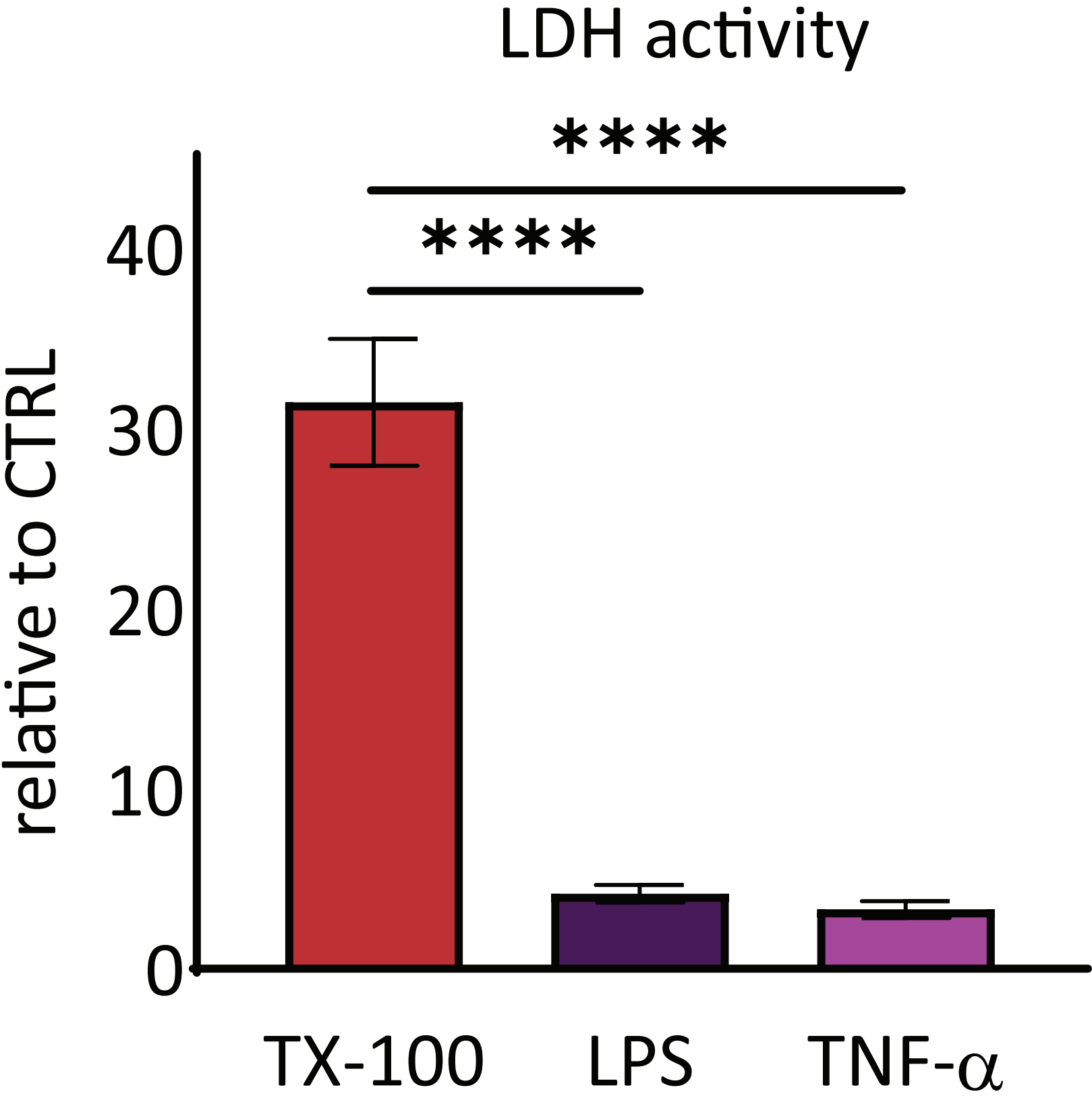
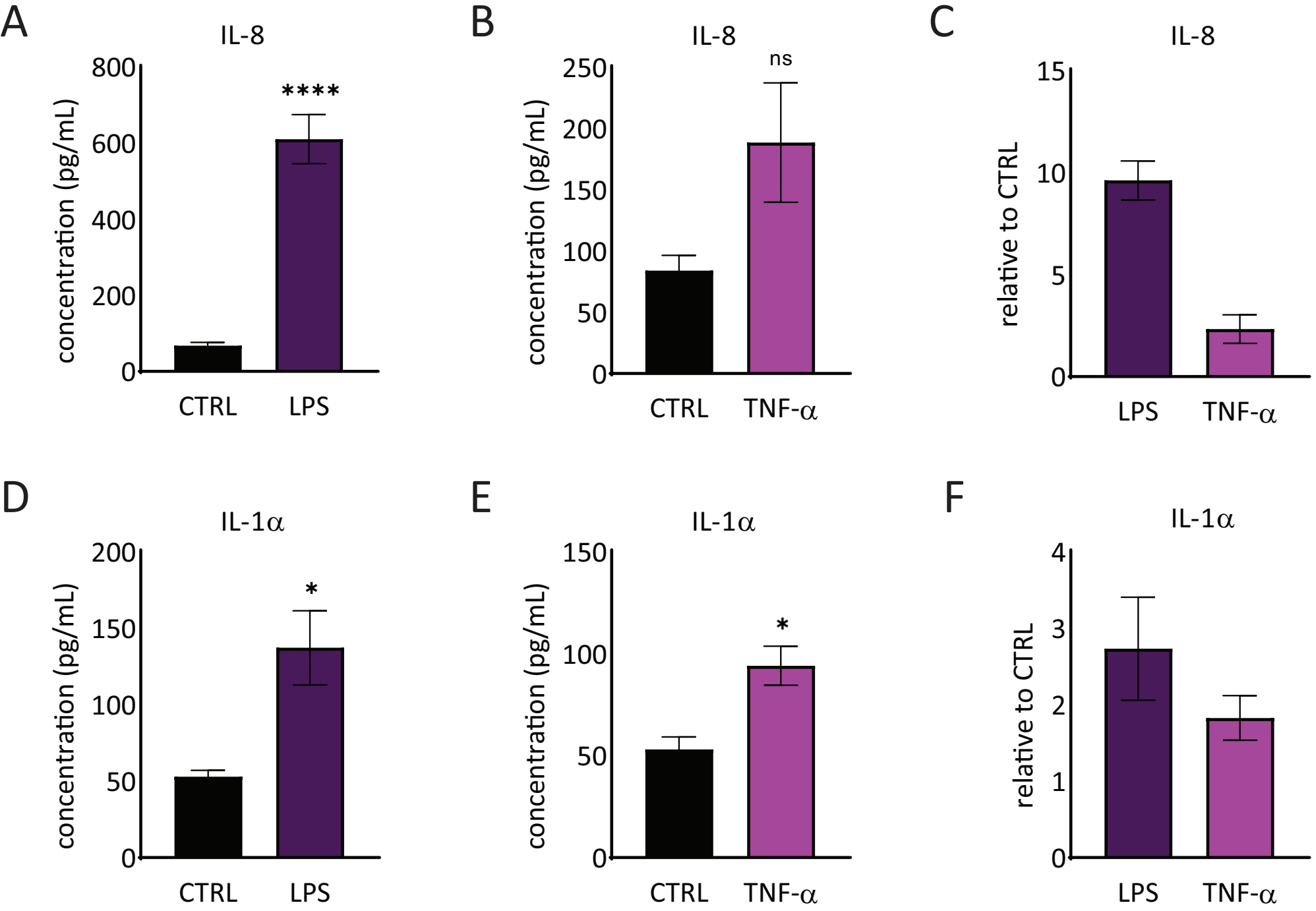


Figure 6



Medium	Medium information	Required quantity
Basal medium	Keratinocyte growth medium + 1 % [v/v] HKGS + 1 % [v/v] 100x antibiotic-antimycotic	36 mL/24 wells
Submerged medium	Keratinocyte growth medium + 1 % [v/v] HKGS + 1 % [v/v] 100x antibiotic-antimycotic + 1.5 mM $CaCl_2$	36 mL/24 wells
ALI medium	Keratinocyte growth medium + 1 % [v/v] HKGS + 1 % [v/v] 100x antibiotic-antimycotic + 1.5 mM $CaCl_2$ + 50 µg/mL ascorbic acid + 10 ng/mL keratinocyte growth factor	216 mL/24 wells

Name of reagents and cells	Company
Acetic acid	Sigma Aldrich
Antibiotic-antimycotic (100x)	Thermo Fisher Scientific (Gibco)
Aqueous Eosin Y Solution	Sigma Aldrich
Calcium chloride	Sigma Aldrich or Merck
DAPI	Roche
Entellan mounting medium	Merck
EpiLife medium (referred to as keratinocyte growth medium)	Thermo Fisher Scientific (Gibco)
Ethanol absolute	Any supplier
Formalin 10%	Leica Biosystems
Human keratinocytes growth supplement (HKGS) (100x)	Thermo Fisher Scientific (Gibco)
Isopropanol	Biosolve B.V.
Kaiser's glycerol gelatine, phenol-free	Merck
Keratinocyte growth factor (KGF)	R&D Systems
Lactate dehydrogenase (LDH) assay kit	Roche
L-ascorbic acid 2-phosphate sesquimagnesium salt hydrate	Sigma-Aldrich
Lipopolysaccharide (LPS), E.coli strain O55:B5	Sigma-Aldrich
Mayer’s Haematoxylin	Sigma-Aldrich
Normal human epidermal keratinocytes (NHEKs)	Lonza
Phosphate-buffered saline	Thermo Fisher Scientific (Gibco)
Recombinant tumor necrosis factor alpha (TNF-α)	ImmunoTools
Sterile ultrapure water	Any supplier
Thiazolyl Blue Tetrazolium Bromide (MTT)	Sigma-Aldrich
Triton X-100	Sigma-Aldrich
Trypan blue (0.4% [v/v])	Any supplier
Trypsin inhibitor	Thermo Fisher Scientific (Gibco)
Trypsin/EDTA (0.05% [v/v])	Thermo Fisher Scientific (Gibco)
Tween 20	Sigma-Aldrich
Xylene	Sigma-Aldrich
Goat Anti-Mouse IgG H&L (Alexa Fluor 488)	Abcam
Tri-sodium citrate dihydrate	Merck

Catalog Number
A6283
15240062
HT110280
C8106 or 1.02378.0500
10236276001
1.07960.0500
MEPI500CA
N/A
3800770
S0015
0016264102BS
1.08635.0100
251-KG-050
4744926001
A8960
L4524
MHS80
00192906
10010056 or 10010-015
11344483
N/A
M5655
93426
N/A
R007100
25300054
P1379
214736
Ab150113
1.06448.0500

Comments/Description
Eosin solution is acidified with 1 mL acetic acid per 100 mL.
Eosin solution is acidified with 1 mL acetic acid per 100 mL.
Prepare a stock solution of 0.144 M, filter sterilize and aliquot in 15 mL tubes and store at -20 °C. It is recommended to prepare new
Stock at 100 µg/mL is prepared. Store aliquots at -20 °C. Working conditon is 1 µg/mL.
Mounting medium for H&E staining.
Contains 0.06 mM of Ca^{2+}.
To reach final concentrations of 0.2% [v/v] BPE, 0.2 ng/mL human recombinant EGF, 0.18 µg/mL hydrocortisone,5 µg/mL bovine transferrin,
Mounting medium for IF staining.
Reconstitute KGF protein at 100 µg/mL in sterile 0.1% [w/v] BSA in PBS. Prepare aliquots and store at -20°C.
Prepare a stock solution of 25 mg/mL, filter sterilize and store aliquots at -20°C. It is recommended to prepare new aliquots every 6 months.
Stock of 1 mg/mL in sterile PBS is prepared. Store aliquots at -20 °C. Working concentration is 100 µg/mL.
Human primary keratinocytes isolated from neonatal foreskin, pooled from at least three donors.
Reference numbers vary from countries, but they are the same product.
Stock of 100 µg/mL in sterile ultrapure water. Working concentration is 40 ng/mL
Stock of 5 mg/mL MTT in sterile PBS is prepared. Working concentration is 1 mg/mL.
For antigen retrieval buffer for IF staining.

Name of material
24-well carrier plate with cell/tissue culture inserts
24-well culture plates
96-well microplates
Centrifuge tubes: 15 and 50 mL
Counting slide two-channel
Glass coverslip
Dispenser pipette tips
Embedding cassettes
Glass pasteur pipettes (230 mm)
Polystyrene serological pipettes: 5, 10, and 25 mL
Sponge for cassettes
Sterile reservoirs, 50 mL
SuperFrost plus microscope slides
T75 cell culture flasks
Tweezers

Company	Catalog Number
Thermo Fisher Scientific (Nunc)	141002
Thermo Fisher Scientific (Nunc)	142475
Any supplier	N/A
Any supplier	N/A
Any supplier	N/A
Any supplier	N/A
Any supplier	N/A
Simport	M490
Any supplier	N/A
Any supplier	N/A
Simport	M476-4
Any supplier	N/A
ThermoFisher Scientific	J1800AMNZ
Thermo Fisher Scientific (Nunc)	156499
Any supplier	N/A

Comments/Description
Inserts: polycarbonate membrane, pore size: 0.4 µm, surface: 0.47 cm^2, pore density: 1 x 10^8 pores/cm^2. Carrier in which the inserts are placed. Well volume: low hanging position, 0.5 mL; medium hanging
Compatible with automated cell counter.

Compatible with microscope slides.

Compatible with stepper pipette. 25 mL tips are recommended.
Compatible with pipette controller.
Compatible with M490 sponges.

Name of equipment	Company	Catalog Number
Aspiration system	Any supplier	N/A
Automated cell counter	Any supplier	N/A
Bright field inverted microscope	Any supplier	N/A
Cell/tissue culture incubator	Any supplier	N/A
Centrifuge	Any supplier	N/A
Dispenser pipette	Any supplier	N/A
Freezer	Any supplier	N/A
Fridge	Any supplier	N/A
Laboratory balance	Any supplier	N/A
Laminar flow hood	Any supplier	N/A
Laser Scanning or Epifluorescence microscope	Any supplier	N/A
Micropipettes 0.5 - 10 µL	Any supplier	N/A
Micropipettes 100 - 1000 µL	Any supplier	N/A
Micropipettes 20 - 200 µL	Any supplier	N/A
Millicell ERS-2 Voltohmmeter	Merck Millipore	MERS00002
Pipette controller	Any supplier	N/A
Vortex	Any supplier	N/A
Water bath	Any supplier	N/A

Comments/Description
To remove the culture medium in a controlled and sterile manner.
To obtain accurate and fast cell and viability counts.
For routinely monitoring of the cell cultures.
To provide a sterile, controlled, and protected environment for cell/tissue cultures. Temperature, CO_2, and humidity controlled.
Provides accurate pipetting and dispensing of liquids.
To store samples or aliquots at -20°C.
To store media and aliquots at 4°C.
To provide a sterile work environment.
Imaging of immunofluorescence stainings.
To pre-warm cell culture reagents to 37°C.

Rebuttal letter

Dear Editor,

This letter accompanies the submission of the revised manuscript entitled “A Reliable Procedure to Cultivate Three-dimensional Reconstructed Human Epidermis at Large Scale” as original manuscript to be published in JoVE.

We would like to thank the reviewers for their helpful comments and suggestions, which helped us to make the content clearer. We have provided answers (marked in green) to all the comments and also marked the changes in the manuscript in green. All the authors have reviewed the revisions and read the manuscript.

Please do not hesitate to contact us if you have any questions or concerns regarding the responses to the reviewers or changes to the manuscript.

We look forward to the manuscript being accepted and published in JoVE.

On behalf of all authors, we thank you for your consideration.

Marc Eeman, PhD

Research Scientist, Dow Silicones Belgium SRL

Editorial comments:

Please note that novelty is not a requirement for publication and reviewer comments questioning the novelty of the article can be disregarded.

You will find Editorial comments and Peer-Review comments listed below. Please read this entire email before making edits to your manuscript.
NOTE: Please include a line-by-line response to each of the editorial and reviewer comments in the form of a letter along with the resubmission.

Please take this opportunity to thoroughly proofread the manuscript to ensure that there are no spelling or grammatical errors.

Protocol Detail: Please note that your protocol will be used to generate the script for the video, and must contain everything that you would like shown in the video. Please ensure that all specific details (e.g. button clicks for software actions, numerical values for settings, etc) have been added to your protocol steps. There should be enough detail in each step to supplement the actions seen in the video so that viewers can easily replicate the protocol.

1) 3.6: Mention magnification.

*The magnification is added in **line 305.***

Protocol Highlight: Please highlight ~ 1.5-2.5 pages of text (which includes headings and spaces) in yellow, to identify which steps should be visualized to tell the most cohesive story of your protocol steps.

1) Starting highlighting from section 4 will make the protocol portion of the video a bit abrupt. Please highlight some steps in section 3 while ensuring continuity.
2) The highlighting must include all relevant details that are required to perform the step. For example, if step 2.5 is highlighted for filming and the details of how to perform the step are given in steps 2.5.1 and 2.5.2, then the sub-steps where the details are provided must be included in the highlighting.
3) The highlighted steps should form a cohesive narrative, that is, there must be a logical flow from one highlighted step to the next.
4) Please highlight complete sentences (not parts of sentences). Include sub-headings and spaces when calculating the final highlighted length.
5) Notes cannot be filmed and should be excluded from highlighting.

We thank the editor for this comment and have added highlights to section 2, 3, and 4.

Section 2.1, 2.2, 3.1-3.17, 4.4,

Discussion: JoVE articles are focused on the methods and the protocol, thus the discussion should be similarly focused. Please ensure that the discussion covers the following in detail and in paragraph form (3-6 paragraphs): 1) modifications and troubleshooting, 2) limitations of the technique, 3) significance with respect to existing methods, 4) future applications and 5) critical steps within the protocol.

Figures:
1) Please replace "µl" with "µL".

Thank you for noticing this typo, it has been adapted in line 530.

Figure/Table Legends: Mention statistical tests used.

''**Figure 5: The cytotoxicity measured via lactate dehydrogenase (LDH) assay.** The data is presented as relative values to CTRL; mean ± SEM, n=9 (TX-100), n=8 (LPS), n=3 (TNF-α). Significance was tested with one-way ANOVA, Dunnett's multiple comparison test. Asterisk denotes statistically significant increase compared to CTRL, ****p < 0.0001).

Figure 6: Proinflammatory reactions in the reconstructed human epidermis. The concentration of IL-8 release of the RhE upon a 24-hour challenge with LPS (**A**) and TNF-α (**B**)**.** Data is represented as mean ± SEM, n=8 (LPS), n=3 (TNF-α). (**C**) Data is represented as the mean of relative value compared to CTRL ± SEM, n=8 (LPS), n=3 (TNF-α). The concentration of IL-1α release of the RhE upon a 24-hour challenge with LPS (**D**) and TNF-α (**E**)**.** Data is represented as mean ± SEM, n=8 (LPS), n=3 (TNF-α). (**F**) Data is represented as the mean of relative value compared to CTRL ± SEM, n=4 (LPS),

n=3 (TNF-α). Significance was tested by an unpaired Student's T-test. Asterisk denotes statistically significant decrease compared to CTRL, *p < 0.05, ****p < 0.0001).''

Commercial Language: JoVE is unable to publish manuscripts containing commercial sounding language, including trademark or registered trademark symbols (TM/R) and the mention of company brand names before an instrument or reagent. Examples of commercial sounding language in your manuscript are EpiLife.

1) Please replace all commercial sounding language in your manuscript with generic names that are not company-specific. All commercial products should be sufficiently referenced in the table of materials/reagents. You may use the generic term followed by "(see table of materials)" to draw the readers' attention to specific commercial names.
2) Please check all figures and numbered tables as well.

Thank you for this comment. The word 'EpiLife' has been changed to 'Keratinocyte growth medium'. This has also been changed in Figure 1 and Table 1.

Table of Materials: Please sort in alphabetical order.

The Table of Materials have been updated and sorted in alphabetical order.

Comments from Peer-Reviewers:

Reviewers' comments:
Reviewer #1:
Manuscript Summary:
This manuscript presents the detailed protocol for production of reconstructed human epidermis. The description is precise, complete, and well explained.

Major Concerns:
None

Minor Concerns:
Several references are provided in multiple copies. The list of references should be deeply revised and checked for its accuracy, repetition, and completeness of information.

Thank you for noticing the reference copies. The reference library was remade and all the references have been checked for accuracy and completeness of information.

Reviewer #2:
Manuscript Summary:
Very good overview of the production of the reconstructed human epidermal tissue. This protocol will be of a major interest for many tissue engineering groups and CROs that wish to use their own in house model
The group provided very detailed guidance that is supported by number of highly relevant references.

Major Concerns:

There is a lack of a classic barrier resistance test (i.e. exposure to the cytotoxic marker like 1% Triton or SDS). The absolute TEER value does not say much about the real barrier properties in the RhE tissues. I have seen very high TEER values, that dropped very quickly after exposure to the cytotoxic markers and in opposite, tissues with relatively low TEER resisted significantly better and produces higher ET-50 values. In addition, this test is one of the requirements of the OECD TG 431 and 439 that describes the use of the Reconstructed human Epidermis (RhE) for the skin irritation and corrosion testing. The group should produce data on at least 3 batches with Triton X-100 (1%) over the time-points of 4,6.8 and 10 hours. Typical dose is 100 microliters per 0.6 cm2 of the tissue. Only this test, in combination with the parameters already presented, will reveal the real characteristics of the tissues.
Along with this, please provide the information on the OD of the Negative controls from the MTT assay (1mg/mL) and ideally establish a range into which the produced lots typically fall in your lab. This is another specification requested by the guidelines that established RhE model for regulatory as well as non-regulatory work.

We thank the reviewer for this comment and fully agree with the fact that the ET-50 values should be presented to reveal the RhE barrier properties. Therefore, the ET-50 assay has been performed in triplicate and the results can be seen in Figure 4B. The OD of the negative controls was on average 1.71 ± 0.073 (mean ± SD, n=3).

''The barrier properties of the RhE model was investigated by assessing both the tissue viability upon topical treatment with a known barrier disruptor and the tissue integrity. The tissue integrity was determined after 15 days by measuring the TEER using a voltohmmeter. The 2567 ± 415 $\Omega.cm^2$ values recorded for the RhEs translate the formation of a continuous barrier (**Figure 4A**). Those values are in range with those reported for reconstituted human epidermal models[1–4]. Additionally, the required exposure time for a cytotoxic reference chemical (i.e., Triton X-100) to reduce the tissue viability by 50% (ET_{50}) was determined with a thiazolyl blue tetrazolium bromide (MTT) assay. The ET_{50} value measured for the RhE was 2.1 hours. This value falls within the acceptance range of other 3D epidermal models that are qualified for reliable prediction of irritation classification (OECD Guideline 439)[5].''

Minor Concerns:
1. The authors should consider to use an abbreviation RhE instead of HEE. RhE is recognized and frequently used abbreviation for the type of the model that is described in this protocol and is even implemented in the OECD Test guidelines.

The authors agree with the comment of Reviewer #2. The abbreviation ''HEE'' has been changed to ''RhE'' in the manuscript (title), figures, and figure and table legends.

2. Was the lipid profile investigated? If so, please add data on this.

The SC lipid profile was not measured of this RhE. However, the authors agree that it would be highly interesting to investigate the SC lipid composition and organization, since a proper SC lipid profile is key for a strong barrier function of the skin.

Reviewer #3:

This protocol describe a method to cultivate human epidermal equivalents (HEE). The protocol is clearly written and instructions for the proposed work are properly described.

1. I however question the novelty as it describes a widely used model that is already described in detail by many groups. Besides the existing mechanistic studies, laboratory protocols with in detail description of how to generate HEE models have also been published based on similar protocol, generating similar models. Unfortunately these are not referenced in the manuscript. More specifically, the herein described culture format (24 wells), culture model (transwell plates with polycarbonate filter allowing air-liquid interface cultures), culture medium (Epilife) and duration (approximately two weeks) have already been established and published on by others.

> *Besides having referenced to several manuscripts of groups that are ahead in the field, the authors agree that several publications have been missing in this manuscript. The added references have been mentioned in the answers to question 3 and 4.*

2. The authors claim the main downsides of in-house HEEs being "the loss of standardization between research institutions and increased labor intensity and time consumption". The aim of the present paper according to the authors is to "address these drawbacks, setting the basis for production at a larger scale. In addition to the abovementioned advantages of in-house HEEs, the current protocol aims to "reduce the intra- and inter-variability among tissues, to reduce contamination risks, and to streamline the cultivation process." The current manuscript however does not provide any new solution to these issues considering that their method is identical to the current protocols used in the field, with equal throughput and cultivation time.

> *We agree with the reviewer, but since JoVE does not require novelty in the topic, the aim of this publication is to support the scientific community scientific community during the process of setting up the cultivation of RhEs in their laboratory. This publication would include the first movie of the full detailed laboratory procedure, which we consider highly valuable for and crucial to the reproduction of this method.*

3. Instead there is a need for new models, with alternative cell sources (preferably not the foreskin keratinocytes as used here, but adult keratinocyte due to their differences in marker expression and response to external stimuli), higher throughput (48wells and up), automated production (bioprinting), lower scale (skin-on-chip) and shorter cultivation period (<7d). Unfortunately, the current paper does not apply to any of these unmet needs, nor does the discussion of the paper addresses any of these concerns.

> *The authors agree with the reviewer and have discussed several of these limitations as follows.*
>
> '' There are several limitations of the RhE model that should be noted. In native human skin there is an equilibrium between the proliferation of keratinocytes in the basal layer and the detachment of corneocytes in the stratum corneum (i.e., desquamation)[6]. However, in vitro, desquamation does not take place. Therefore, the

corneocytes remain attached to the RhE, and form a thick stratum corneum that is less physiologically relevant. Hence, there is a limited cultivation timespan of RhEs. Moreover, this RhE model is made simple and straightforward, since it consists of a singular cell type, i.e. the keratinocyte, which is the most abundant cell type of the epidermis. However, there are other cell types resident in the epidermis, such as melanocytes, dendritic cells (i.e., Langerhans cells), T cells (e.g., $CD8^+$ cells), and Merkel cells[7]. To enhance the physiological relevance of the skin model, researchers have made skin models more complex by adding melanocytes[8] , immune cells[9], or patient-derived cells[10]. One should keep in mind that the barrier properties of human skin models are different compared to native human skin, due to notably a different SC lipid composition and a higher barrier permeability [11–15]. However, several studies have reported on changes in barrier properties of human skin models by the cultivation under hypoxia[16] or decreased relative humidity[17], the modulation of the dermal matrix with Chitosan[18], and alteration in the free fatty acids in the culture medium[19]. Moreover, in both simple and more complex RhEs, the culture conditions and medium composition can be modulated to mimic pathological features. By challenging the model with cytokines, an abnormal morphology[20] and alterations in gene and protein expression levels can be established that are typically observed in common skin disorders, such as atopic dermatitis and psoriasis[21–26]. Silencing specific genes in NHEKs prior to seeding of the RhEs, is another approach used to mimic features of skin disorders and investigate new therapeutic solutions[27, 28]. Besides modeling an epidermal layer only, a dermal compartment can be included in the model (i.e., named human skin equivalents or full-thickness models) by embedding fibroblasts in a collagen matrix prior to RhE reconstruction, making it more physiologically relevant and suitable for aging and wound healing related studies[29–31]. Additionally, tumor spheroids have been added to human skin equivalents to study melanoma progression[32, 33]. The latest advances in the field of skin models are bio-printing and skin-on-a-chip. Multiple research groups have succeeded recently in the development of (perfusable) bio-printed skin equivalents[34–36]. The proposed protocol takes advantage of a 24-well format and a carrier plate, avoiding inserts to be handled individually. However, the study scale is still quite limited and lacks automation. By implementing the use of bio-printing or skin-on-a-chip, smaller skin models can be used with more automated processes and on a larger scale.''

4. Furthermore, the citations of the paper are strongly focussed on toxicology research, citing papers of rather low impact compared to available literature in leading journals of the field. Although I am not at liberty to ask for citing specific work from specific research groups, the authors may want to consider any of the examples below of (recent) work that could be worth mentioning: 1. Consensus opinion on quality parameters for 3D skin models in skin barrier research. 2. Detailed laboratory protocols for construction of HEE cultures from primary and immortalized keratinocytes. 3. HEE models using primary (patient-derived) keratinocytes, including inflammatory/sensitization molecule stimulation and host responses. 4. Effects of culture conditions to HEE structure and functions.

The authors agree that several references to more recent work should be included in this manuscript, that are also focused on mechanistic research. Therefore, the following references have been added to the already existing references in the manuscript on this

topic. Additionally, multiple references have been added in the paragraph of limitations, in the previous answer.

> ''In addition to commercially available 3D skin models multiple research groups have developed their own RhEs[22, 37–41].''

> ''There are also numerous other assays developed that utilize commercial RhE models, to evaluate phototoxicity[42], to test drug formulations[43], cosmetic formulations and active ingredients[44], to study the skin barrier function[45] and to test the biological response to environmental stressors[46–49].''

Additionally, several parameters of the characterization of our RhE model, have been compared with the parameters described in the publication of Van den Bogaard and colleagues in JID (2020).

> '' The barrier properties of the RhE model was investigated by assessing both the tissue viability upon topical treatment with a known barrier disruptor and the tissue integrity. The tissue integrity was determined after 15 days by measuring the TEER using a voltohmmeter. The 2567 ± 415 $\Omega.cm^2$ values recorded for the RhEs translate the formation of a continuous barrier (**Figure 4A**). Those values are in range with those reported for reconstituted human epidermal models[1–4]. Additionally, the required exposure time for a cytotoxic reference chemical (i.e., Triton X-100) to reduce the tissue viability by 50% (ET_{50}) was determined with a thiazolyl blue tetrazolium bromide (MTT) assay. The ET_{50} value measured for the RhE was 2.1 hours. This value falls within the acceptance range of other 3D epidermal models that are qualified for reliable prediction of irritation classification (OECD Guideline 439)[5].''

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Author Biography Dijkhoff

-Studied Bio-pharmaceutical sciences at Leiden University, Leiden, the Netherlands.

-Focused on visualization of lipid processing enzymes in the skin and resulted in a publication in Leiden at the department of drug delivery technology under supervision of Prof. Joke Bouwstra.

-Last part of the master focused on the validation of novel targets for inflammatory bowel disease with high-troughput co-cultures at Galapagos, Leiden.

-Position as Associate Scientist at Galapagos, mainly working on target validation for pulmonary fibrosis and Hepatitis B. Developed CRISPR CAS9 cell lines for target validation.

- Currently part of the CITYCARE project, funded by the European Commission under the Horizon 2020 Marie Skłodowska-Curie Action. She is working and enrolled at the University of Fribourg in Switzerland for her doctoral degree. Her research aims at understanding the effects of diesel exhaust and solar radiation on of skin to provide innovative solutions for a better skin protection.

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Roxane holds both a chemical engineering diploma from CPE Lyon and a MSc in Formulation Science from the University of Lille. She had the opportunity to gain experience as a chemist in the academic sector at the University of Cardiff as well as in the industrial sector at Arkema Inc. (King of Prussia, USA) and BASF (Shanghai, China). During her MSc thesis, she studied the oxidation of sebum upon diesel exhaust exposure. She is currently enrolled as a Marie Curie early stage researcher at the department of Biomedical Sciences and Specialty Surgical Sciences at the University of Ferrara in Italy. Her doctoral research investigates the impact of cigarette smoke on both the cutaneous responses and biomechanical properties of healthy and compromised skin models.

Giuseppe Valacchi, PhD

Giuseppe Valacchi obtained his B.S. with Laude in physiology, MS in physiopathology and his PhD degree in Cell Physiology and Neuroimmuno-physiology at the University of Siena. During his training he has worked as “exchange PhD student" in the “Department of Molecular and Cell Biology at the University of California at Berkeley" where he started out some studies on the effect of environmental oxidative stress such as O3 and UV on skin physiology. After his PhD graduation, he continued working as Post Doc at the University of Berkeley until December 2000. Between 2000 and 2004 he was appointed first as Post Doc in the “Department of Internal Medicine" and then in the "Department of Nutrition" at the University of California at Davis (UCD). In 2005 he become Faculty and member of the Academic Federation in the Department of Internal Medicine (UCD). At the end of 2006 he was awarded with the “progetto rientro dei cervelli”, granted by the Italian Ministry of Health and returned to Italy at the University of Siena as Assistant Professor until 2011. Since 2011 he is appointed as Associate Professor in Physiology at the University of Ferrara. From 2008 is also Adjunt Prof. at Kyung Hee University, Seoul, South Korea and starting from August 2016 he is Associate Professor in Regenerative Medicine at North Caroline State University. His research has been focused in understanding the cellular, and molecular mechanisms that define the tissues physio-pathological responses to altered redox homeostasis. He is a member of the SFRR Europe Council and he is author of more than 235 peer reviewed internationals papers, 15 book chapters, one book. He has been invited speaker to more than 100 international conferences and organizer/Chair to over 50 international meetings. He has won several awards among them the Entelligence Award from Actelion, OCC Young Investigator Award, Science and Education Award and recently the Exposome grant. In 2018 he was awarded with the “Doctorate Honoris Causa” in Biochemistry and Pharmacy from the University of Buenos Aires for his work in the redox biology field. He is the Associate Editor of several international journals among which is Mediators of Inflammation, Frontiers in Cellular Biochemistry, World Research Journal of Biochemistry, Journal of Complementary and Traditional Medicine, Biomed Research International (Dermatology Subjects), Oxidative Medicine and Cellular Longevity; in addition he is member of the Editorial Board of several journals such as Genes and Nutrition, Open Biochemistry Journal, Archives in Biophysics and Biochemistry, Clinical Immunology Endocrine & Metabolic Drugs, Frontiers in Inflammation Pharmacology, Clinical Anti-Inflammatory & Anti-Allergy Drugs, BioFactors, Cosmetics.

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Prof. Dr. Barbara Rothen-Rutishauser has received her Ph.D. in 1996 in cell biology at the Swiss Federal Institute of Technology (ETH) in Zurich. From 1996 to 2000 she held a post-doctoral position in Biopharmacy at the Institute of Pharmaceutical Sciences at the ETH and in 2000 she joined Prof. Peter Gehr's research group at the University of Bern, Switzerland as a postdoc. After promotion to group leader in 2006 she completed her habilitation in cell biology in 2009. B. Rothen-Rutishauser is an expert in the field of cell-nanoparticle interactions in the lung, with a special focus on 3D lung cell models and various microscopy techniques such as laser scanning and transmission electron microscopy. Since 2011 she is the new chair in BioNanomaterials at the Adolphe Merkle Institute, University of Fribourg, Switzerland, the position is shared equally with Prof. Alke Fink. The research group's activities stretch over many fields from material synthesis and characterization to biological responses and hazard assessment. Prof. Rothen-Rutishauser has published more than 220 peer-reviewed papers and is an associate editor of the journal "Particle and Fibre Toxicology".

MARC EEMAN – RESEARCH SCIENTIST

Professional Background

- Joint PhD degree in Agricultural Sciences and Biological Engineering from University of Liège, Faculty of Gembloux Agro-Bio Tech (Belgium) & Lund University (Sweden)
- Study of the molecular interactions of antimicrobial lipopeptides with skin extracellular lipid matrix
- Worked for 2 years at StratiCELL, a Belgian SME/CRO for in vitro objectivation of cosmetics

Current Role/Research Interests

- Research Scientist, Home and Personal Care
- Seneffe (Dow Silicones Belgium) since 2010
- Development of in vitro testing to create claims for Dow' portfolio of cosmetic actives and polymers for skin care
- Research interests: In vitro cell culture, signaling pathways, molecular interactions, biophysical techniques

Current Projects

- Coordinator of a EU-funded research program aiming at investigating the effects of environmental pollution on skin biological and biomechanical properties (4 academic partners, 3 PhD students)
- Evaluation of potential benefits of anti-ageing actives