Video Article

Generation of a Three-dimensional Full Thickness Skin Equivalent and Automated Wounding

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Abstract

In vitro models are a cost effective and ethical alternative to study cutaneous wound healing processes. Moreover, by using human cells, these models reflect the human wound situation better than animal models. Although two-dimensional models are widely used to investigate processes such as cellular migration and proliferation, models that are more complex are required to gain a deeper knowledge about wound healing. Besides a suitable model system, the generation of precise and reproducible wounds is crucial to ensure comparable results between different test runs. In this study, the generation of a three-dimensional full thickness skin equivalent to study wound healing is shown. The dermal part of the models is comprised of human dermal fibroblast embedded in a rat-tail collagen type I hydrogel. Following the inoculation with human epidermal keratinocytes and consequent culture at the air-liquid interface, a multilayered epidermis is formed on top of the models. To study the wound healing process, we additionally developed an automated wounding device, which generates standardized wounds in a sterile atmosphere.

Video Link

The video component of this article can be found at https://www.jove.com/video/52576/

Introduction

The skin is the largest organ of the body. It creates a barrier between the external environment and the internal organs. Moreover, the skin protects the body from fluid loss, environmental influences, injuries and infections and helps to regulate body temperature[1]. Due to its exposed location, the skin is often affected by mechanical, thermal or chemical trauma. Though the skin is generally capable of self-repair, multiple local factors such as infection, oxygenation, and venous sufficiency can lead to impaired wound healing. Wound healing can also be interfered by systemic factors as obesity, alcoholism, smoking, medication, nutrition and diseases such as diabetes.[2]

The process of wound healing can be divided into 3 phases: (i) the inflammatory phase, (ii) the proliferative and the (iii) remodeling phase. Upon injury to the skin, a complex signal-cascade starts, leading to the closure of the wound.[3] After injury, the wound is bleeding and a blood clot is formed. Fibroblasts move into the blood clot and replace it with new tissue which is subsequently remodeled over years.

The current understanding of the biological processes underlying cutaneous repair is limited. Small animal and pig models have been used to study wound healing. However, these results cannot be directly transferred to humans due to species-specific differences. In addition to these *in vivo* models, some aspects of wound healing can be studied by simulating a wound situation via scratching of *in vitro* monolayer cultures based on immortalized cell lines or primary cells.[4] These scratching models are highly standardized but do not sufficiently reflect the complex *in vivo* physiology.[5] Besides two-dimensional models, three-dimensional human skin equivalents have been developed for dermatological research. The dermal part of these models are generated using various scaffolds including decellularized dermis,[6] collagen hydrogels,[7,8] glycosaminoglycans[9] or synthetic materials.[10] Employing these skin equivalents, the role of epithelial-mesenchymal interactions[11], the re-epithelialization, the cellular crosstalk between fibroblasts and keratinocytes and the influence of different growth factors can be studied. Moreover, these models are useful to gain new knowledge about how fibroblasts migrate into the wounded area and how chemotactic factors influence tissue regeneration.[12]

Not only generation of the wound healing model itself is challenging, but also to establish a highly standardized wound in a model is problematic. Common techniques to create wounds are scratch tests,[13] burns,[14] tape abrasion,[15] thermal injury,[16] suction blisters,[17] liquid nitrogen,[18] lasers,[19] scalpels,[18] meshers[6] and biopsy punches.[20] Most of these methods have the same pitfalls. Injuries implemented manually are difficult to standardize and to reproduce between multiple tests. Size, shape and depth of the wound vary between studies and thus impair the quality of

research data. The use of laser for defined skin wounding can be relatively easily standardized but leads to a situation mimicking burn wounds. Heat applied by laser can cause protein denaturation, platelets aggregation or vessels constriction, which can lead to necrotic tissue.

In an alternative approach, we developed an automated wounding device (aWD), which allows us to generate defined and precise cutaneous wounds under sterile conditions. Wounding parameters, like depth and speed of penetration as well as revolutions of the drill head can be regulated. In this study, we combined the aWD with in house developed full thickness skin equivalents (ftSE) that are comparable to a protocol published by Gangatirkar *et al.* [8] The dermal layer of the skin equivalent is composed of human dermal fibroblasts (hDF), which are embedded in a collagen I hydrogel. On the dermal layer, human epidermal keratinocytes (hEK) are seeded. Within two weeks at the air-liquid interface the hEK build up an epidermis composed of several vital cell layers and a *stratum corneum*. Besides the generation of this model, this study shows the use of the aWD to create defined and precise wounds in ftSE.

Protocol

NOTE: The protocol is designed for the production of 24 full thickness skin equivalents. Human dermal fibroblasts and epidermal keratinocytes were isolated from skin biopsies according to a previously published protocol.[21,22] Informed consent was obtained beforehand and the study was approved by the institutional ethics committee on human research of the Julius-Maximilians-University Würzburg (vote 182/10).

1. Production of Dermal Component

1. Dissolve collagen with 0.1% acetic acid to a final concentration of 6 mg/ml. For the gel neutralization solution, mix 232.5 ml 2x DMEM, 7.5 ml fetal calf serum, 7.5 ml 3 M HEPES, 2.5 ml chondroitin sulfate (5 mg/ml). Keep gel neutralization solution and collagen solution on ice. Calculate 500 µl of gel per insert/skin equivalent.
   NOTE: As the gel neutralization solution will be mixed with two parts of collagen solution prepare double amount of collagen.
2. Place 24 inserts into a 24 well plate.
3. Resuspend human dermal fibroblasts (hDF) in 10 ml DMEM, centrifuge for 5 min at 270 x g and count the cells. Extract the needed amount of hDF (5 x 10^4 cells per 500 µl collagen gel) and centrifuge the cells for 5 min at 270 x g.
4. Remove the supernatant and carefully resuspend the hDF in 4 ml cooled gel neutralization solution without producing air bubbles. From this step make sure to work as fast as possible to avoid premature solidifying of the collagen gels.
5. Resuspend the solution with 8 ml of collagen solution.
6. Take the collagen-cell-mixture with a multistep-pipette and fill quickly 500 µl of mixture in each insert. Incubate the gels for 20 min in an incubator (37 °C, 5% CO_2) to jell the gels.
7. Submerge the gels in 2 ml Dulbecco's Modified Eagle Medium (DMEM) + 10% fetal calf serum (FCS) per well and incubate it for 24 hr in an incubator (37 °C, 5% CO_2).

2. Addition of Human Epidermal Keratinocytes (hEK)

1. Remove medium after 24 hr. Dissolve fibronectin human protein with ultrapure water to a final concentration of 50 µg/ml. Cover each dermal equivalent with 25 µl of fibronectin solution. Incubate the gels for 30 min in an incubator (37 °C, 5% CO_2).
2. In the meantime, resuspend the hEK in 10 ml KGM-2 and set cell concentration to 1 x 10^6 cells/ml with KGM-2 ready + 5% FCS. Seed 1 x 10^5 hEK on each gel. Incubate gels for 45 min in the incubator (37 °C, 5% CO_2) to allow cells to adhere.
3. Submerse the gels with KGM-2 ready + 5% FCS and culture them in the incubator (37 °C, 5% CO_2).

3. Culture of Full Thickness Skin Equivalents (ftSE)

1. Culture ftSE for 6-7 days in descending FCS-concentration (5%, 2%, and 0% FCS). Change the medium every 2-3 days with the next lower FCS concentration. For this, completely remove the medium and add 1.6 ml of fresh medium.
2. On day 7, remove medium completely.
   NOTE: Do not touch the ftSE surface with the tip of pipette while doing so.
3. Place every insert into 1 well of a 6 well plate with sterile forceps. Add 1.5 ml airlift medium per well, ensure not to wet the ftSE surface. Change medium every 2-3 days for additional 14 days.
   NOTE: The filling level of the medium is up to the meniscus of the ftSE.

4. Histological Analysis

1. Fixing and paraffin-embedding of the ftSE
   1. Remove culture medium, transfer inserts in fresh 24 well plates and fix tissues by adding 1.6 ml of 4% paraformaldehyde to each well and incubate the tissues for 2 hr at RT. Caution! Paraformaldehyd is toxic, manipulate under fume hood. Transfer the ftSE to a tissue-embedding cassette. Remove remaining fixative by washing with water and dehydrate the tissue using ascending ethanol concentrations.
   2. Divide the skin equivalent by a vertical cut in the middle. Place the two pieces in a paraffin-filled metal base mold with cut surfaces downwards. Add the tissue cassette on top of the mold as a backing.
   3. Cut 3-5 µm slices and float them on a 40 °C water bath for straitening, mount slides onto suitable microscope slides. Dry slices thoroughly.

2. Hematoxylin & Eosin (H&E) and immunohistochemical (IHC) staining
   1. Place slides into rack and incubate for 1 hr at 60 °C. Make sure that paraffin is melted. Stain rehydrated slices with H&E as a standardized overview staining.

2. For H&E staining prepare 4x glass staining cuvettes with xylol, 3x with ethanol 96%, 2x with ethanol 70%, 1x with ethanol 50%, 4x deionized water, 1x hematoxylin, 1x HCl-ethanol (13.7 ml 1 M HCl ad 200 ml 50% ethanol), 1x tab water, 1x eosin (1 g eosin in 100 ml deionized water) and 2x 2-propanol.
3. Place slides 10 min in xylol I and 10 min in xylol II to deparaffinize and rehydrate slides. Dip slides 3x in ethanol 96% I, 3x in ethanol 96% II, 3x in ethanol 70% and 3x in ethanol 50%. Rotate slides in deionized water. To differentiate hematoxylin dye, dip 2x in HCl-ethanol. Rinse in deionized water. For blueing, place slides 5 min in tab water. For eosin staining, place slides 1 min in eosin and wash afterwards in deionized water. For dehydration, dip slides 2x in ethanol 70%, 2x in ethanol 96% and place them for 5 min in 2-propanol I, for 5 min in 2-propanol II, 5 min in xylol I and 5 min in xylol II. After H&E staining, cell nuclei are stained in blue, cytoplasm and extracellular matrix are stained in red.
4. For antigen retrieval, remove paraffin with xylene and rehydrate slices for staining. Place slices in steam cooker and cook deparaffinized and rehydrated slices for 20 min in pre-heated 10 mM citrate buffer (pH 6).
5. Place slides in washing buffer (PBS buffer + 0.5% Polysorbat 20) and circle slices with a liquid repellent slide marker pen or diamond stylus.
6. Place slides in a moisture chamber. Block endogenous peroxidase, by adding 100 µl 3% hydrogen peroxide solution onto each slice. Caution! Very hazardous in case of skin and eye contact. Handle with personal protective equipment. Wash slides in washing buffer.
7. Apply primary antibody and incubate for 1 hr at RT. Wash slides three times with washing buffer. From here on, samples should be kept in the dark.
8. Use biotin-streptavidin detection system for detection of specific antigen-antibody binding.
9. Counterstain nuclei with Hematoxylin for 1 min. Dehydrate and mount slides.
10. Image samples using an inverse microscope.

5. Injury by Use of the Automated Wounding Device (aWD)

1. Prepare the aWD by disinfecting the working area with 70% ethanol and ultraviolet light. Make sure the drilling head of the desired size (*e.g.,* 1 mm) is attached.
2. Place skin equivalents into designated areas of the autoclaved sample carrier plate using sterile forceps. Place the sample carrier plate into the socket below the drilling head.
3. Use the control software to set wounding parameters as follows: spin velocity: 15,000 rpm; penetration: 1.5 mm; propulsion: 100 Hz. These parameters need to be defined for each sample type individually. Transfer wounding parameters to the aWD.
4. Start wounding procedure. Remove sample carrier plate from the socket. Transfer wounded ftSE back into inserts used for culture using sterile forceps.
5. Proceed with culture of wounded ftSE in the incubator (37 °C, 5% CO_2) for investigation of regeneration. Alternatively, use the models for histological analysis at any time.

Representative Results

The isolated hDF and hEK differ both in morphology and in expression of typical markers. The hDF showed typical spindle-shape morphology, whereas morphology of hEK can be described by a cobblestone morphology. The cells were characterized by immunohistochemical staining (**Figure 1**) before using them for ftSE. The hDF are positive for vimentin (**Figure 1A**), a marker for fibroblasts. Primary hEK highly express early differentiation protein cytokeratin-14 (**Figure 1B**) but nearly no late keratinocyte differentiation protein cytokeratin-10 (**Figure 1C**).

Following primary culture, we detached the cells from the cell culture flasks and used them for the generation of ftSE. The ftSE were cultured for 7 days under submers medium conditions (**Figure 2A**) followed by a 14 days culture at the air-liquid interface (**Figure 2B**). During this process, the ftSE contract significantly. Within 21 days the hEK are proliferating (**Figure 2C-E**). By changing the medium level in such way that the surface of the models is exposed to air and by increasing calcium concentration, the hEK are stimulated to differentiate into a multi-cellular epidermis that is composed of several vital cell layers of keratinocytes in different differentiation states and a *stratum corneum* (**Figure 2E**).

Comparing the Haematoxylin & Eosin staining of ftSE (**Figure 2E**) with native human skin (**Figure 2F**) it becomes obvious that ftSE mimics histological architecture of the skin. This can be further demonstrated by immunohistochemical stainings (**Figure 3**). The hDF in the collagen I hydrogel can be stained with primary antibodies against vimentin (**Figure 3A, E**). Keratinocytes form an epidermal layer composed of cytokeratin-14 positive cells (**Figure 3B, F**) and the late differentiation marker cytokeratin-10 is expressed in the supra-basal layers (**Figure 3C, G**). Furthermore, the *stratum corneum* is positive for filaggrin (**Figure 3D, H**).

Following 21 day culture period, the aWD was used to generate cutaneous wounds in the ftSE. As shown in **Figure 4A**, the aWD is constructed as a closed box-system to ensure sterile conditions. In **Figure 4B**, a schematic drawing of the wounding process is demonstrated. The ftSE are fixed on a sample carrier plate. The whole wounding process, including the setting of parameters such as lowering speed and revolutions of the drill head (**Figure 4D**) as well as the depth of penetration is controlled computer assisted. The wounding procedure can be monitored optically via a front window or by a high resolution camera that records all steps during the wounding procedure. Size, shape and depth of the wound injury can be adjusted individually (**Figure 4C**).

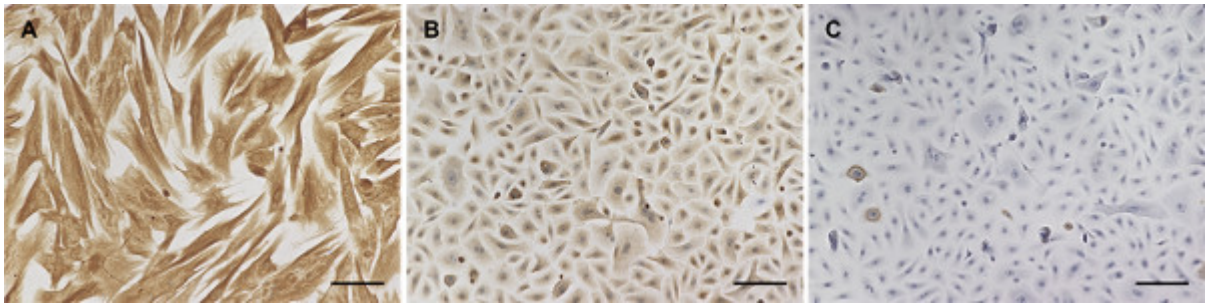

Figure 1. Characterization of cells used for the construction of full thickness skin equivalents. Immunohistochemical staining of primary human dermal fibroblasts for vimentin (**A**) and of epidermal keratinocytes for the early differentiation keratin filament cytokeratin-14 (**B**) and the late differentiation filament cytokeratin-10 (**C**). Positive staining is shown by brown color. Scale bars indicate 100 µm. Please click here to view a larger version of this figure.

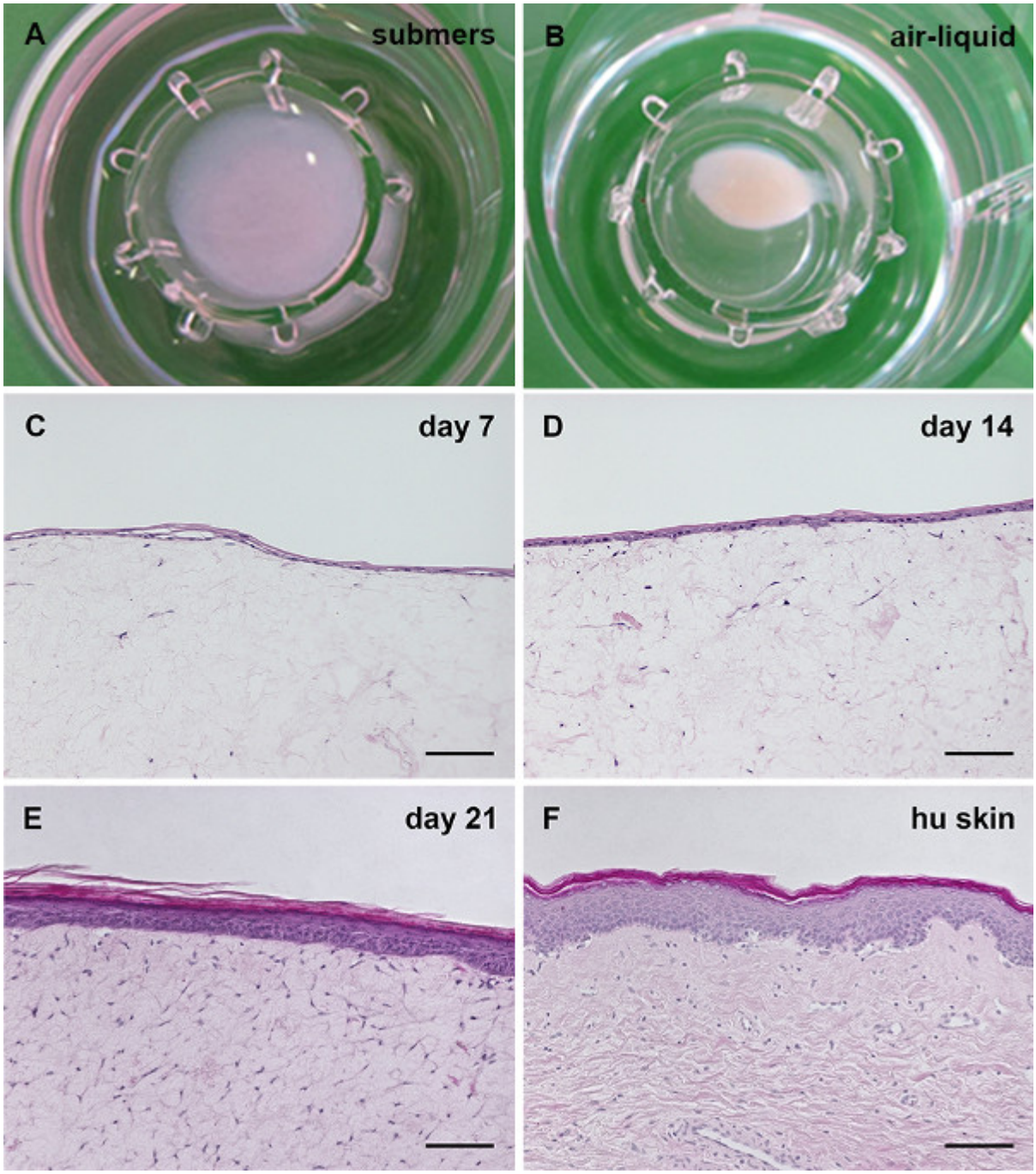

Figure 2. Changes of culture conditions and maturation of full thickness skin equivalents. Macroscopic pictures of full thickness skin equivalents cultured for 7 days under submers conditions (**A**) and 14 days at the air-liquid interface (**B**). Comparison of Haematoxylin & Eosin staining of full thickness skin equivalents in different developmental stages: After 7 days (**C**), 14 days (7 days at the air-liquid interface, **D**) and 21 days (14 days at the air-liquid interface, **E**). Haematoxylin & Eosin staining of native human skin (**F**). Scale bars indicate 100 µm. Please click here to view a larger version of this figure.

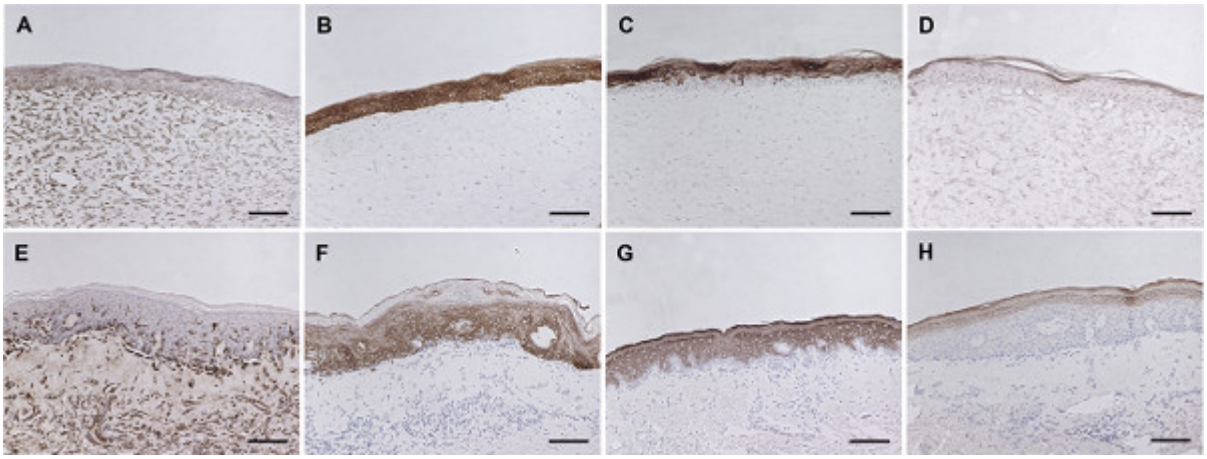

Figure 3. Characterization of full thickness skin equivalents. Comparison of immunohistochemical staining of full thickness skin equivalents (**A**-**D**) and native human skin (**E**-**H**) for vimentin (**A**, **E**), cytokeratin-14 (**B**, **F**), cytokeratin-10 (**C**, **G**) and filaggrin (**D**, **H**). Scale bars indicate 100 µm. Please click here to view a larger version of this figure.

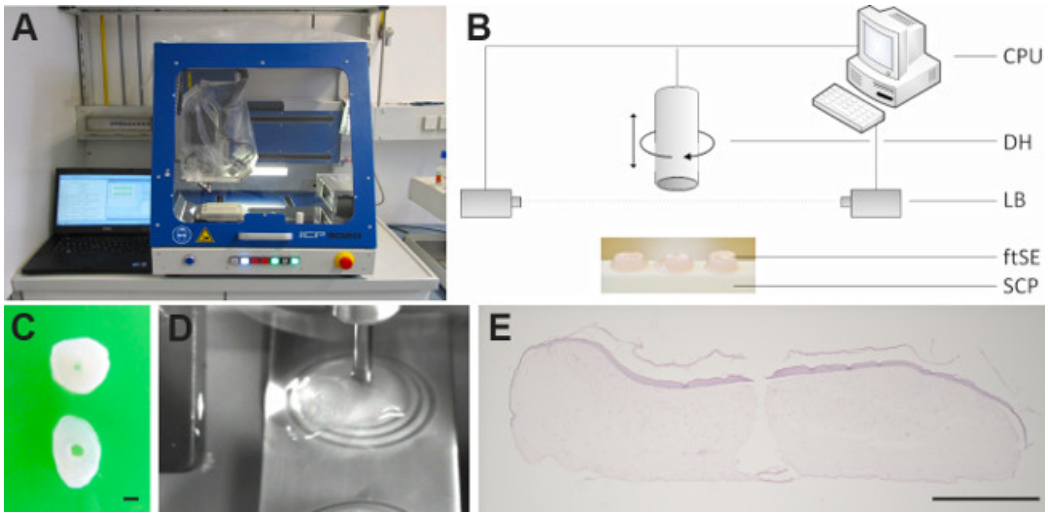

Figure 4. Automatic Wounding Device for controlled wounding on full-thickness skin equivalents. The automated wounding device (aWD) provides sterile conditions during the controlled wounding procedure (**A**). An attached computer (CPU) controls the aWD. The full thickness skin equivalents (ftSEs) are placed on the sample carrier plate (SCP), deviations in sample morphology are equalized by a light barrier (LB), which triggers the wounding procedure. The wound shape depends on the used drilling head (DH). The diameter ranges from 1 mm (**C**, upper image) to 2 mm (**C**, lower image). The wounding procedure is monitored and can be recorded for documentation and quality management (**D**). Hematoxylin & Eosin stained cross section of a full thickness skin equivalent wounded with the automated wounding device using a 1 mm drilling head (**E**). All scale bars indicate 2 mm. Please click here to view a larger version of this figure.

Discussion

In vitro cells are usually expanded in two-dimensional cell cultures, in which cells adhere to plastic surfaces. However, these culture conditions do not reflect the physiological three-dimensional conditions in which cells grow *in vivo*. Under three-dimensional conditions, cells can form natural cell-cell and cell-matrix attachments and migrate in three-dimensions. Especially in the cutaneous wound healing the resemblance of the *in vivo* situation is pivotal to generate meaningful data, as cell migration and matrix generation are key elements of wound healing.

In order to mimic these conditions we developed ftSE that are composed of human primary cells and reflect both the dermal and the epidermal layer of the skin. During two weeks of culture at the air-liquid interface, the hEK form an epidermis composed of several layers of keratinocytes in different differentiation phases and a *stratum corneum*. Furthermore, the dermal layer is composed of hDF, which are embedded in a collagen I hydrogel. During the culture time, the hDF remodel the dermal component, which results in a significant shrinkage of the models (**Figure 2B**).

To prepare reproducible and standardized wounds, we developed an aWD. The computer controlled machine can set defined and precise cutaneous injuries (**Figure 4**). Depending on the intended research, depth, shape and size of the injury can be adapted. However, due to the technical specifications of the aWD and the morphology of the ftSE, it is recommended to set a minimal penetration of 100 µm reproducibility. The aWD is operating under sterile conditions in a closed box-system and all components can be sterilized by autoclaving, disinfection solutions or ultraviolet light. In preparation for the wounding procedure the samples need to be transferred to a sample carrier plate. This plate facilitates an exact position of the samples. Furthermore the adhesion between sample and the sample carrier plate is sufficient to keep the samples in place during the wounding procedure. The wounding procedure itself can be monitored optically via a front window or by a high resolution

camera that records all steps from a close-up position. Unlike other systems that use lasers to excavate tissue to generate a wound, the aWD employs a rotating drill. Thus, no heat is applied during the procedure, which is a vital perquisite to investigate mechanical wounds. Additionally, specific shape of the drilling head ensures a material removal from the wound channel. Using empirically determined wounding parameters mentioned above the wounding procedure can be adjusted to meet specific physiological properties of either the ftSE or native skin samples. This way any undesirable side effects like unintentional detachment of epidermal layers can be minimized and reproducible wounding performed with a success rate of 90%.

This study demonstrates that the developed ftSE partly mimic the *in vivo* situation of skin accurately and thus can be employed as a model for wound healing studies. Though important aspects of the wound healing process such as the effect of the vasculature, the blood clot and the immune system are lacking in the model, the epidermal-mesenchymal and cell-matrix interaction is recapitulated in a three-dimensional highly standardized environment. In addition, we could show that the aWD generates standardized wounds in skin models. In combination, both technologies can be used to investigate wound healing and the effect of substances and drugs that support the healing process. In order to mimic the *in vivo* situation more closely, the ftSE can be extended by other cells such as melanocytes, skin tumor cells or micro-vascular endothelial cells.

Disclosures

The authors have nothing to disclose.

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