Organotypic in vitro models of human cutaneous squamous cell carcinoma

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Replacement of animal-derived collagen matrix by human fibroblast-derived dermal matrix for human skin equivalent products

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Abstract

Reconstructed human skin equivalents (HSEs) are representative models of human skin and widely used for research purposes and clinical applications. Traditional methods to generate HSEs are based on the seeding of human keratinocytes onto three-dimensional human fibroblast-populated non-human collagen matrices. Current HSEs have a limited lifespan of approximately 8 weeks, rendering them unsuitable for long-term studies. Here we present a new generation of HSEs being fully composed of human components and which can be cultured up to 20 weeks. This model is generated on a primary human fibroblast-derived dermal matrix. Pro-collagen type I secretion by human fibroblasts stabilized during long-term culture, providing a continuous and functional human dermal matrix. In contrast to rat-tail collagen-based HSEs, the present fibroblast-derived matrix-based HSEs contain more continuity in the number of viable cell layers in long-term cultures. In addition, these new skin models exhibit normal differentiation and proliferation, based on expression of keratins K10/K15, and K16/K17, respectively. Detection of collagen types IV and VII and laminin 332 was confined to the epidermal–dermal junction, as in native skin. The presence of hemidesmosomes and anchoring fibrils was demonstrated by electron microscopy. Finally, we show that the presented HSE contained a higher concentration of the normal moisturizing factor compared to rat-tail collagen-based skin models, providing a further representation of functional normal human skin *in vitro*. This study, therefore, demonstrates the role of the dermal microenvironment on epidermal regeneration and lifespan *in vitro*.

Introduction

Human skin equivalents (HSEs), also designated as organotypic co-cultures, are three-dimensional culture systems that are generated by seeding human keratinocytes onto a dermal equivalent that is usually seeded with human fibroblasts. Under specific culture conditions, a HSE is formed that shows high similarity with the native tissue from which it was derived.[1-6] HSEs have the advantage that they can harbor different cell types (keratinocytes, melanocytes, fibroblasts, etcetera) that are surrounded by a local microenvironment resembling the *in vivo* situation, and in which cellular processes are normalized compared to conventional monolayer cultures. HSEs are an attractive tool for pharmacotoxicological testing and to study cell–cell, cell–matrix and dermal–epidermal interactions. However, current HSEs have a limited lifespan of approximately 8 weeks due to high contractility of the dermal matrix and resulting poor epidermal homeostasis, rendering them unsuitable for long-term studies. Long-lived HSEs that can be cultured beyond 8 weeks would allow for studies focusing on the effects of long-term exposition to various exogenous stressors. Also, the effects on skin homeostasis and skin cancer by application of physiologically low doses of chemicals or substances can be studied in long-lived skin models in contrast to skin models with a limited lifespan of 8 weeks.

Furthermore, the quality of current HSEs may be compromised by the fact that they are largely based on non-human dermal equivalents e.g. collagen originating from rat or bovine tendon, and bovine fibrin. These non-human biopolymers allow for successful HSE formation, but they still render the dermal compartment an incomplete approach to human extracellular matrix (ECM) *in vivo*, which contains lipids, fibrin, glycosaminoglycans and proteoglycans in addition to collagen as its main substituent. Although the use of human de-epidermized dermis (DED) may offer a human alternative to these animal-derived dermal equivalents, the application of this method is limited by uncontrollable variation in DED thickness and low availability of the native tissue. In addition, all dermal equivalents mentioned serve as exogenous scaffolds that need to be seeded with fibroblasts in order to provide a microenvironment permissive of HSE formation, thereby increasing their artificial character. In contrast, the generation of long-lived HSEs presented here is based on human fibroblast-derived matrices (FDMs) as dermal equivalents.

During tissue homeostasis and repair, fibroblasts synthesize, organize and maintain connective tissue through ECM production and remodeling. This essential function of fibroblasts can be utilized *in vitro* to produce solely cell-based dermal equivalents. *In vitro* modulation of various culture conditions resulted in a microenvironment enabling the

production of FDMs.[7-9] In these cultures, multilayered human fibroblasts are stimulated towards high ECM accumulation, fibrillar fibronectin organization, and the formation of actin stress fibers.[10] The main stimulating additive in these cultures is ascorbic acid, which promotes the *in vitro* processing of pro-collagen to collagen a-chains. The morphological, biochemical and physical characteristics of the resulting FDMs are superior to those of fibroblast-populated reconstituted ECMs.[7, 9] This is supported by the fact that collagen fibril bundle assembly is featured by ECM biosynthesis, since fibroblasts seeded in isolated biopolymers do not form collagen fibril bundles whereas fibroblasts producing their own matrix do form these important structural elements of a fully functional ECM.[8] Although the development of isolated FDMs was established in earlier research as mentioned above, by seeding keratinocytes on these FDMs, a new generation of long-lived HSEs is established, harboring only human cell-derived components. This change in the local microenvironment of these FDM-based HSEs has the potential to improve cell–matrix interactions and maintenance of a functional stem cell compartment, rendering the full human HSEs suitable for long-term studies *in vitro*. We here present a comparison between the newly developed long-lived FDM-based HSEs and established HSEs, focusing on their morphology, ECM production, differentiation and proliferation, ultrastructural basement membrane formation, barrier function and lifespan.

Methods

Cell culture

For isolation of normal human epidermal keratinocytes (NHEKs), epidermis was obtained through overnight incubation of fresh mamma reduction surplus skin with dispase II (Roche Diagnostics, Almere, the Netherlands). NHEKs were isolated from the epidermis through incubation with trypsin at 37°C for 15 min. After trypsin inactivation, the cells were filtered using a 70 µm cell strainer (BD Biosciences, Breda, the Netherlands), and cultured in keratinocyte medium at 37°C and 7.3% CO_2 until subconfluency. Keratinocyte medium consisted of 3 parts Dulbecco's modified Eagle's medium (DMEM, Gibco/Invitrogen, Breda, the Netherlands) and 1 part Ham's F12 medium supplemented with 5% fetal bovine serum (FBS, HyClone/Greiner, Nürtingen, Germany), 0.5 µM hydrocortisone, 1 µM isoproterenol, 0.1 µM insulin (Sigma–Aldrich, Zwijndrecht, the Netherlands), 100 U/ml penicillin and 100 mg/ml streptomycin (Invitrogen, Breda, the Netherlands). In all experiments, secondary cultures were used. For isolation of normal human dermal fibroblasts (NHDFs), human dermis was obtained through overnight incubation of fresh mamma reduction surplus skin with dispase II. NHDFs were isolated from the dermis by incubation with a solution consisting of collagenase II (Invitrogen) and dispase II (ratio 1:3 and 3 ml/g dermis) at 37°C for 2h. The cells were filtered using a 70 µm

cell strainer, and cultured in fibroblast medium at 37°C and 5% CO_2 until subconfluency. Fibroblast medium consisted of DMEM supplemented with 5% FBS, 100 U/ml penicillin and 100 mg/ml streptomycin. Culture medium was refreshed every 3 days. In all experiments, secondary or tertiary cultures were used.

Dermal equivalents

Fibroblast-derived matrices (FDMs) were generated by seeding $6x10^5$ NHDFs into polyester permeable supports (6 well plates with 0.4 µm pore size Transwell inserts, Corning Incorporated, Schiphol-Rijk, the Netherlands). The FDMs were cultured for 3 weeks in fibroblast medium supplemented with 100 mg/ml ascorbic acid phosphate (Sigma–Aldrich) under submerged conditions and the culture medium was refreshed every 2 days. Rat-tail collagen matrices were constructed as described earlier [3]. In brief, acetic acid extracted rattail collagen was seeded with $1.2x10^5$ NHDFs on the same polyester permeable supports as the FDMs and incubated for a period of 1 week with unsupplemented fibroblast medium under submerged conditions.

Human skin equivalents

Human skin equivalents were generated by seeding $4x10^5$ NHEKs onto the FDMs and NHDF-seeded rat-tail collagen matrices that were prepared in advance. Cultures were incubated overnight in keratinocyte medium as described above, supplemented with 1% FBS, 53 µM selenious acid, 10 µM L-serine, 10 µM L-carnitine, 1 µM DL-α-tocopherol-acetate, 100 mg/ml ascorbic acid phosphate, $2.4x10^5$ M bovine serum albumin and a lipid supplement containing 25 µM palmitic acid, 15 µM linoleic acid and 7 µM arachidonic acid (Sigma–Aldrich). The cultures were then lifted to the air–liquid interface in supplemented keratinocyte medium as described above, except for the fact that FBS was omitted, the concentration of linoleic acid was increased to 30 µM and ascorbic acid phosphate was halved to 50 mg/ml. Medium was refreshed every 2 days. FDM-based HSEs were processed for analysis after 1, 2, 3, 4, 6, 8, 13, 18 and 20 weeks of air-exposed culture. Rat-tail collagen-based HSEs were processed for analysis after 2 and 8 weeks of air-exposed culture.

Protein determination by enzyme-linked immunosorbent assay

Quantification of human pro-collagen type I in the culture medium of FDM-based HSEs was performed by enzyme-linked immunosorbent assay (MetraCICP ELISA kit; Quidel Corporations, CA, USA). Culture medium of FDM-based HSEs was collected at each refreshment throughout the entire culture period, starting at the moment of fibroblast seeding, 3 weeks prior to seeding of keratinocytes. Measurements and data analysis were performed according to the manufacturer's procedure. The data are expressed as ng/ml

culture medium with standard deviations resulting from triplicate measurements of specimens originating from three independent cultures.

Morphological and immunohistochemical analyses

All cultures were washed in PBS. One half of each culture was snap-frozen in liquid nitrogen while the other half was fixed in 4% paraformaldehyde, dehydrated and paraffin embedded. Global morphological analysis was performed on 5 µm formalin-fixed paraffin-embedded sections through staining with haematoxylin and eosin (HE). Immunohistochemical analysis of keratins 10, 15, 16 and 17, involucrin, Ki67, laminin 332 and collagen types IV and VII, was performed on 5 µm frozen sections of the FDM-based HSEs, which were air dried overnight and fixed in acetone for 10 min. Specifications of the primary antibodies used in this study are listed in Table 1. After incubation with primary antibodies, sections were processed as previously described using the biotin–streptavidin system.[11] Secondary antibodies included FITC-conjugated goat anti-mouse IgG (Dako Cytomation, Glostrup, Denmark) and Cy3-conjugated goat anti-mouse IgG (Jackson ImmunoResearch Laboratories, Cambridgeshire, UK). All sections were counterstained with 40,6-diamidino-2-phenylindole, dihydrochloride (DAPI; Molecular Probes, Invitrogen). Sections were mounted with Vectashield (Brunschwig chemie, Amsterdam, the Netherlands) and coverslipped.

Estimation of proliferation index

The proliferation index was determined by counting the number of Ki67-positive nuclei among the total number of basal cells. A minimum of 100 cells were counted at immunofluoresence level (magnification 200×) at three different regions in each section. The resulting data are expressed as the mean of two independent experiments ±SD.

Table 1 | Primary anitbodies used for immunohistochemical staining of tissue sections.

Antigen (clone)	Source*
K10 (DE-K10)	ICN Biomedicals Inc., Ohio, USA
K15 (LHK 15)	Neomarkers, Fremont, USA
K16 (LLOO25)	Dr. I.M. Leigh, London, England
K17 (CK-E3)	Sigma, Saint Louis, Missouri, USA
Involucrin (SY5)	Sanbio B.V. Uden, The Netherlands
Ki67 AB-2 (Mb67)	Neomarkers, Union, CA, USA
laminin 332 (MB165)	Dr. M. Aumailley, Cologne, Germany
Type IV collagen (PHM12)	Chemicon, Temecula, USA
Type VII collagen (LH7.2)	Dr. I.M. Leigh, London, England

* Antibodies not purchased from indicated sources were personal gifts from the investigator named.

Electron microscopy

Cultures were cut into small blocks of 1 mm^3 and fixed in 2% paraformaldehyde/2.5% glutaraldehyde in 0.1M sodium cacodylate buffer with pH 7.4. After fixation for 1h at room temperature, specimens were rinsed in PBS 3 times, and post-fixed in 1% osmiumtetroxide in cacodylate buffer, pH 7.4 for 1h at 4 °C. After rinsing 3 times in PBS specimens were dehydrated in an ethanol series up to 100% and embedded in epoxy resin LX-112. Ultrathin sections were prepared, and after staining with uranylacetate and lead hydroxide, sections were observed and recorded using a Philips CEM10 electron microscope (Philips, Eindhoven, the Netherlands).

Semi-quantitative measurement of natural moisturizing factor in the stratum corneum

Using confocal Raman spectroscopy, the molecular concentration profile of natural moisturizing factor (NMF) in the stratum corneum of the HSEs was measured semi-quantitatively. All experiments have been performed by RiverDiagnostics (Rotterdam, the Netherlands). In brief, 1 cm^2 fragments of both HSEs cultured for 8 weeks and normal human skin samples were placed with their stratum corneum touching the surface of the measurement window of the skin analyzer. Optimal spectral acquisition times and depth ranges were determined carefully. Per skin sample, a minimum of 10 Raman depth profiles were measured. Per volunteer or skin model (n=2) a minimum of ten profiles were measured per location. Data analysis was performed by RiverDiagnosts and discussed with our laboratory. Analyses were focused on extracting information regarding molecular NMF concentration profiles in the stratum corneum. The profile was represented in ASCII tables containing a depth axis and relative molecular concentrations. The profile data were represented in Microsoft Excel graphs to compare the relative composition at various depths between cultured HSEs and native human skin. The Raman spectrophotometer had a spectral range of 400–4500 cm^{-1} and a spectral resolution <5 cm^{-1}. The depth resolution was <5 µm and the sensitivity signal throughput from skin to detector was >50% over the entire spectral range.

Statistics

Statistical significance was determined using two-tailed Student's *t* test.

Results

Microenvironment in the dermal matrix

In HSEs generated with rat-tail collagen as the main constituent of the dermal compartment, NHDFs are surrounded by a high concentration (3.2 mg/ml) of collagen type I and collagen fibers, while in HSEs generated with fibroblast-derived matrix (FDM), NHDFs are surrounded by their own secreted ECM (Figure 1). To evaluate the effect of the altered microenvironment, different aspects of these skin models were compared. First, we have evaluated the number of viable cell layers during the culture period. After 2 weeks of culture, differences were observed in the number of viable cell layers, as FDM-based HSEs consisted of 4–5 viable cell layers (Figure 2a), while rat-tail collagen-based HSEs consisted of 7–8 viable cell layers (Figure 2d). In both skin models the stratum basale, stratum granulosum, stratum spinosum and the stratum corneum were present. A remarkable observation was done in long-term cultures of these models. After 13 weeks of culture, the number of viable cell layers in human FDM-based HSEs remained constant at the initial level (3–5 cell layers, Figure 2b) and decreased to approximately 3 cell layers after

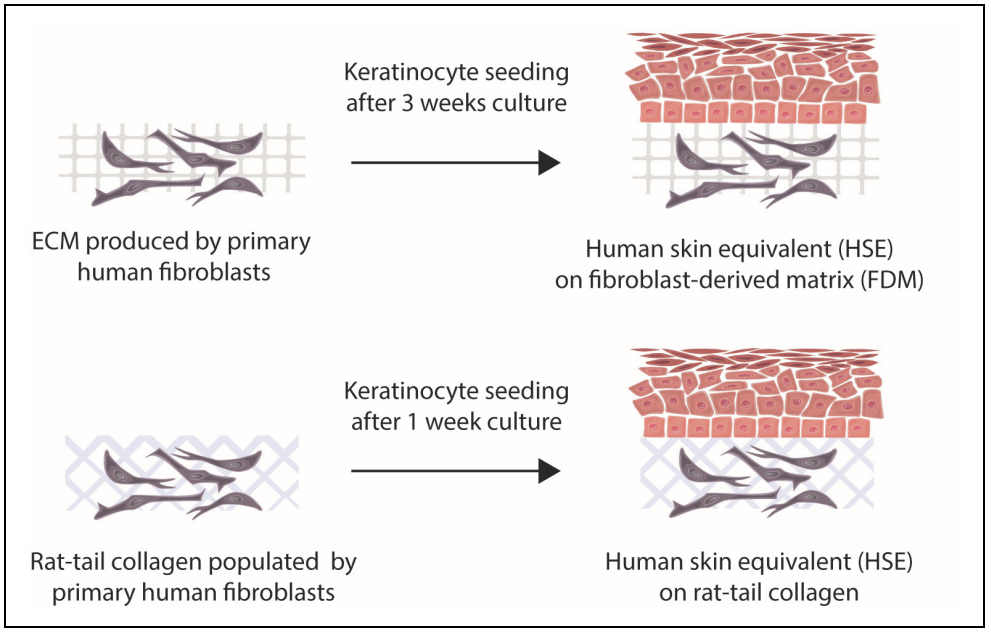

Figure 1 | Schematic representation of the two human skin equivalents (HSEs) investigated in this study. HSEs based on human fibroblast-derived extracellular matrix (ECM) are cultured 3 weeks prior to seeding the keratinocytes, while HSEs based on rat-tail collagen are seeded only 1 week prior to seeding the keratinocytes. After culturing the HSEs at the air–liquid interface, a fully stratified epidermis is formed irrespective of the dermal matrix.

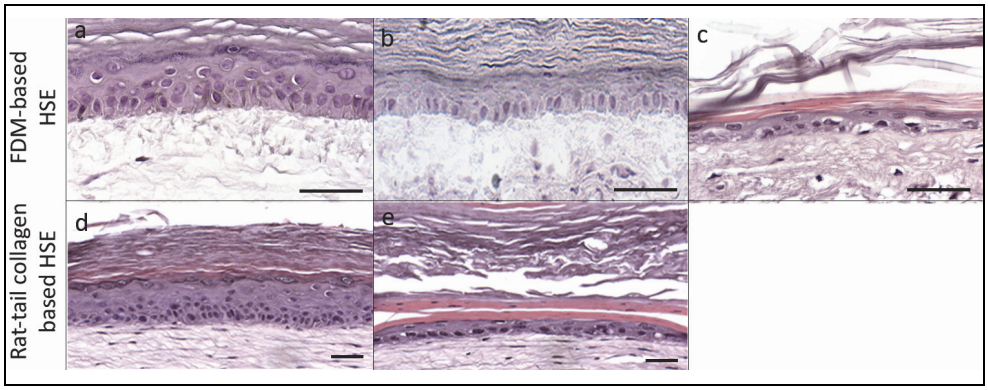

Figure 2 | Epidermal lifespan is modulated by the microenvironment in the dermal matrix. Human keratinocytes were cultured at the air–liquid interface on fibroblast-derived matrices (a–c) or rat-tail collagen matrices populated with fibroblasts (d, e) for 2 weeks (a, d), 8 weeks (e), 13 weeks (b) or 20 weeks (c). Haematoxylin–eosin (HE) stained cross-sections demonstrate the differences in epidermal thickness in culture time. Scale bar: 25 µm.

20 weeks of culture (Figure 2c). Rat-tail collagen-based HSEs cultured for 8 weeks showed a decrease in the number of viable cell layers (4–5 cell layers, Figure 2e) but could not be cultured beyond 8 weeks. These observations indicate that the dermal microenvironment influences epidermal lifespan *in vitro*.

Pro-collagen type I excretion in fibroblast-derived matrix-based HSEs

Pro-collagen type I was measured in culture medium collected during the first 8 weeks of FDM-based HSEs in the absence of growth factors. NHEKs were added after 3 weeks. Measurements were performed 2 weeks prior to NHEK seeding, at the moment of NHEK seeding and 2, 4, 6 and 8 weeks after NHEK seeding. A significant increase in pro-collagen type I secretion was measured during the culture period up to the fourth week (945 ±72 ng/ml), after which the production decreased to the level prior to seeding the keratinocytes (week 0) (284 ±32 ng/ml) (Figure 3). From this experiment we concluded that after three weeks of FDM preparation sufficient pro-collagen (233 ±7 ng/ml) has been secreted by the NHDFs to form a dermal equivalent on which NHEKs could be successfully seeded.

Keratinocyte proliferation in fibroblast-derived matrix-based HSEs

The expression of hyperproliferation-associated protein keratins 16 and 17 (K16, K17) was assessed to evaluate the activation status of the epidermis generated on fibroblast-derived matrices. These proteins were not detected in FDM-based HSEs irrespective of the

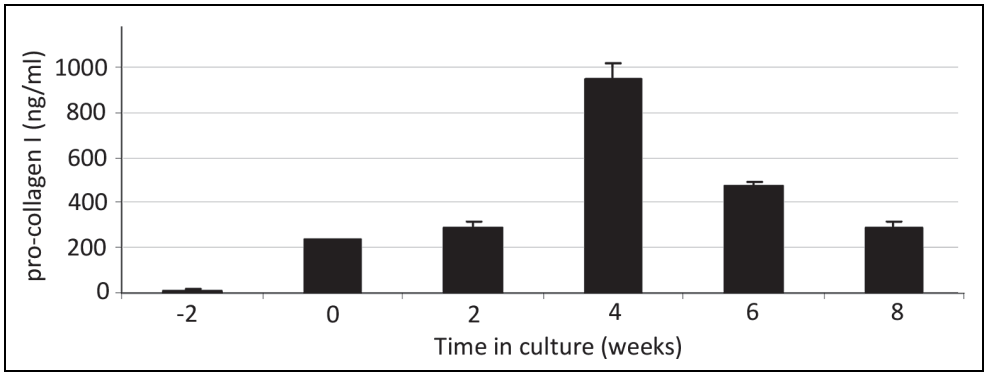

Figure 3 | Quantification of pro-collagen type I secretion into the culture medium of fibroblast-derived matrix (FDM)-based human skin equivalents (HSEs). Concentrations of pro-collagen I in the culture medium were determined by ELISA 2 weeks prior to keratinocyte seeding (t=-2), at keratinocyte seeding (t=0) and 2, 4 6 and 8 weeks after keratinocyte seeding. Bars represent means with standard deviations of triplicate measurements performed in three independent cultures.

culture period (Figure 4a). However, at the periphery of these cultures, K16 and K17 are expressed irrespective of the culture period, indicating cellular activity in migrating keratinocytes (Figure 4b). In previous studies we have shown that the abovementioned proteins K16 and K17 are similarly expressed in rat-tail collagen-based HSEs when they are cultured up to 4 weeks.[12] In addition, we have shown that the expression of these markers was modulated by the number of NHDFs incorporated in the rat-tail collagen matrix.[3] In order to further monitor the proliferation status of the NHEKs cultured on FDMs, we evaluated the proliferation index, expressed as the number of Ki67-positive cells in the basal cell layer of FDM-based HSEs. After 1 week of culture, the proliferation index was 15 ±7%, which decreased to 12±4% in the fourth week of culture, which is similar as in native tissue (12 ±3%) (Fig. 5) and rat-tail collagen-based HSEs.[12] After the fourth week, the proliferation index further decreased and remained stable around 5 ±3% in weeks 6, 8 and 13, to decrease to 3 ±1% after 18 weeks (Fig. 5).

Differentiation in fibroblast-derived matrix-based HSEs

To examine whether epidermal differentiation is affected by the dermal microenvironment, the presence of various protein markers specific for keratinocyte differentiation has been monitored by immunohistochemistry throughout the culture period of the FDMbased HSEs. Expression of the early differentiation marker keratin 10

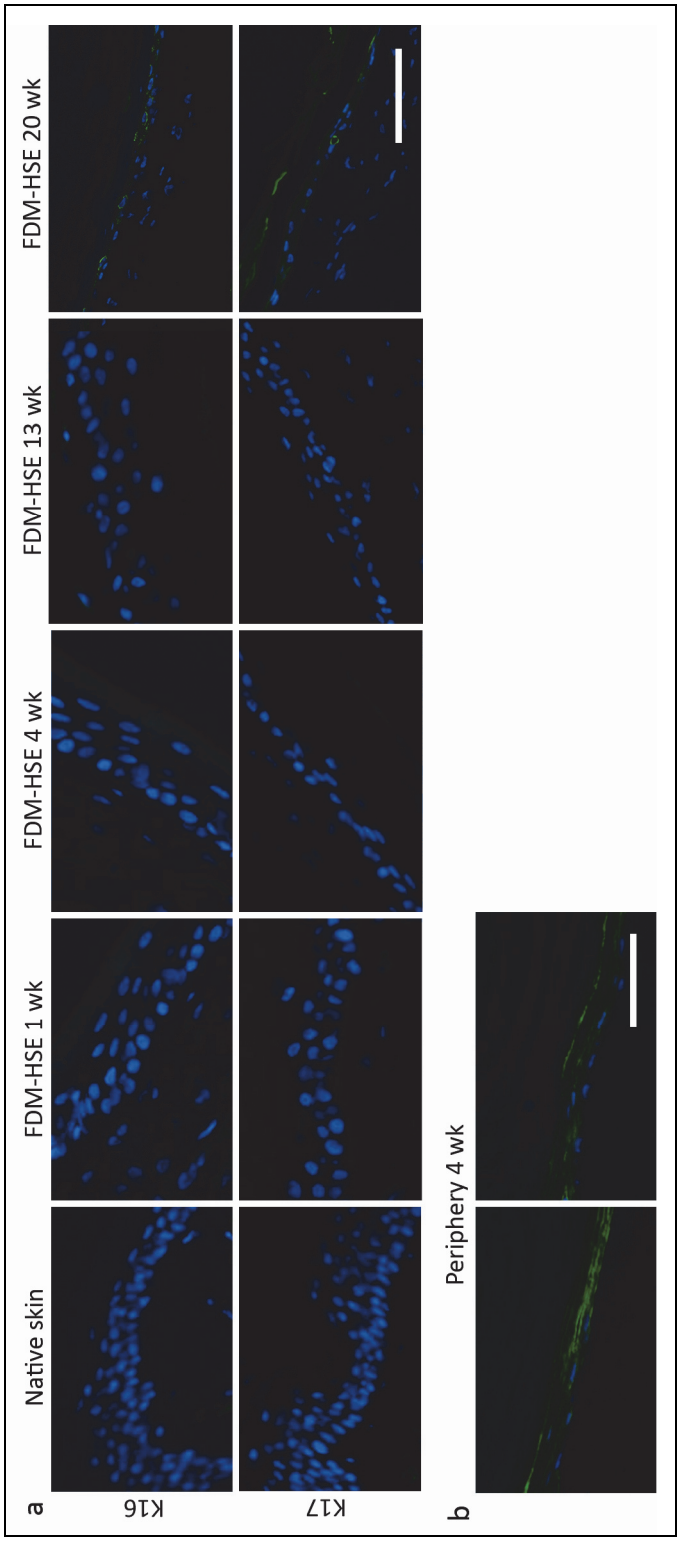

Figure 4 | Immunohistochemical detection of hyperproliferation-associated markers in fibroblast-derived matrix (FDM) based human skin equivalents (HSEs) cultured for longer periods. Human keratinocytes were cultured for 1, 4, 13 and 20 weeks at the air–liquid interface on FDMs. Immunofluorescence staining on cross-sections demonstrate that K16 and K17 are virtually absent in native skin and in FDM-based HSEs cultured for longer periods up to 20 weeks (a). At the periphery of the cultures these keratins are expressed irrespective of culture period, as shown in the periphery of 4-week old cultures (b). Scale bars: 25 µm.

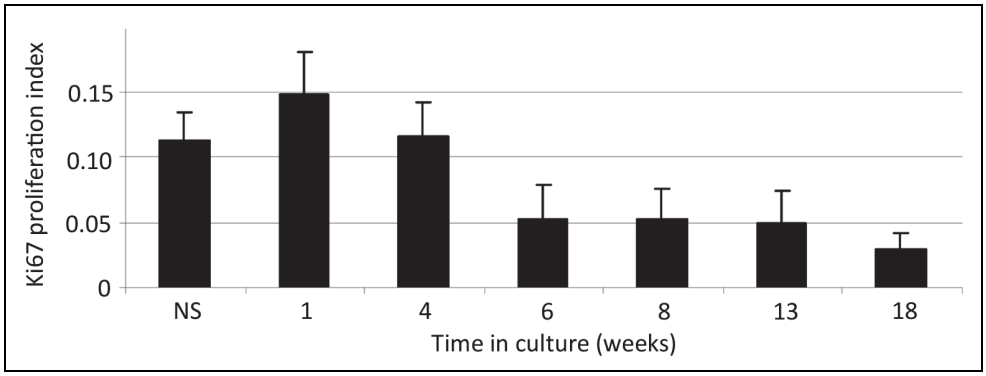

Figure 5 | Detection of proliferation-specific marker Ki67 in human fibroblast-derived matrix (FDM) based human skin equivalents (HSEs). Human keratinocytes were cultured for 1, 4, 6, 8, 13 and 18 weeks at the air–liquid interface on FDMs. The percentage of Ki67-positive cells in these cultures was determined by counting the number of Ki67-positive cells among the total number of basal cells. The resulting data are expressed as the mean of two independent cultures (±SD).

(K10) was only observed in the suprabasal cell layers throughout the entire culture period up to 13 weeks, similar to the native situation (Figure 6) and rat-tail collagen-based HSEs.[3] The basal epithelial marker keratin 15 (K15) was expressed in all basal cell layers as found in native tissue and rat-tail collagen-based HSEs,[13] while the expression of the terminal differentiation marker involucrin changed during culture (Figure 6). Involucrin is normally expressed in the stratum granulosum of native skin and rat-tail collagen-based HSEs,[3] as observed in FDM-based HSEs cultured for 1 week. However, involucrin expression shifted also to the stratum spinosum when FDM-based HSEs were cultured up to 13 weeks or longer (Figure 6).

Basement membrane composition in fibroblast-derived matrix-based HSEs

To evaluate to what extent the basement membrane is formed in FDM-based HSEs, we performed transmission electron microscopy of FDM-based HSEs that were cultured for 13 weeks and performed protein staining of collagen types IV and VII and laminin 332. The transmission electron microscopy recording revealed that the human fibroblasts synthesized new collagen fibers and that the dermal–epidermal junctions exhibit a continuous basement membrane with lamina lucida, lamina densa, regular hemidesmosomes and anchoring fibers (Figure 7). Immunoflurescence analysis showed

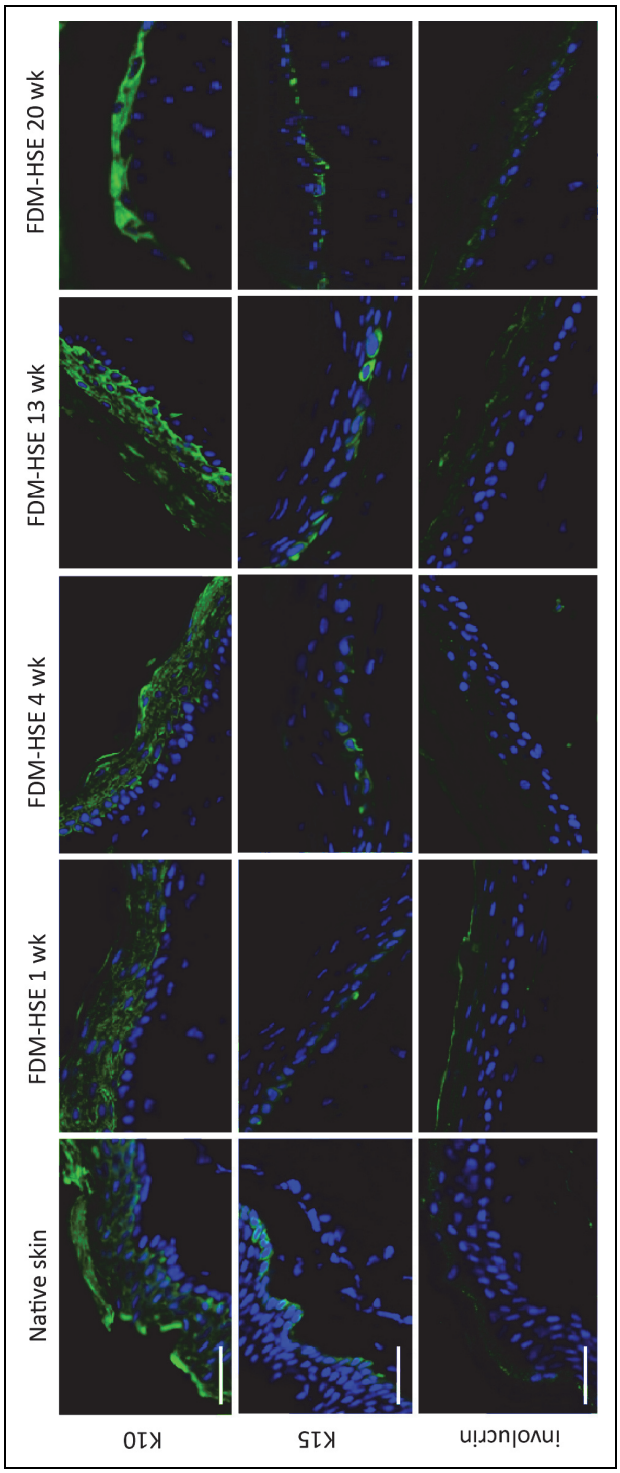

Figure 6 | Expression of differentiation-specific markers in fibroblast-derived matrix (FDM) based human skin equivalents (HSEs) cultured for longer periods. Human keratinocytes were cultured for 1, 4, 13 and 20 weeks at the air–liquid interface on FDMs. Immunofluorescence staining on cross-sections demonstrate that K10 and K15 are expressed as in native skin and that involucrin expression is altered in long-term cultures (13 weeks). Scale bars: 25 µm.

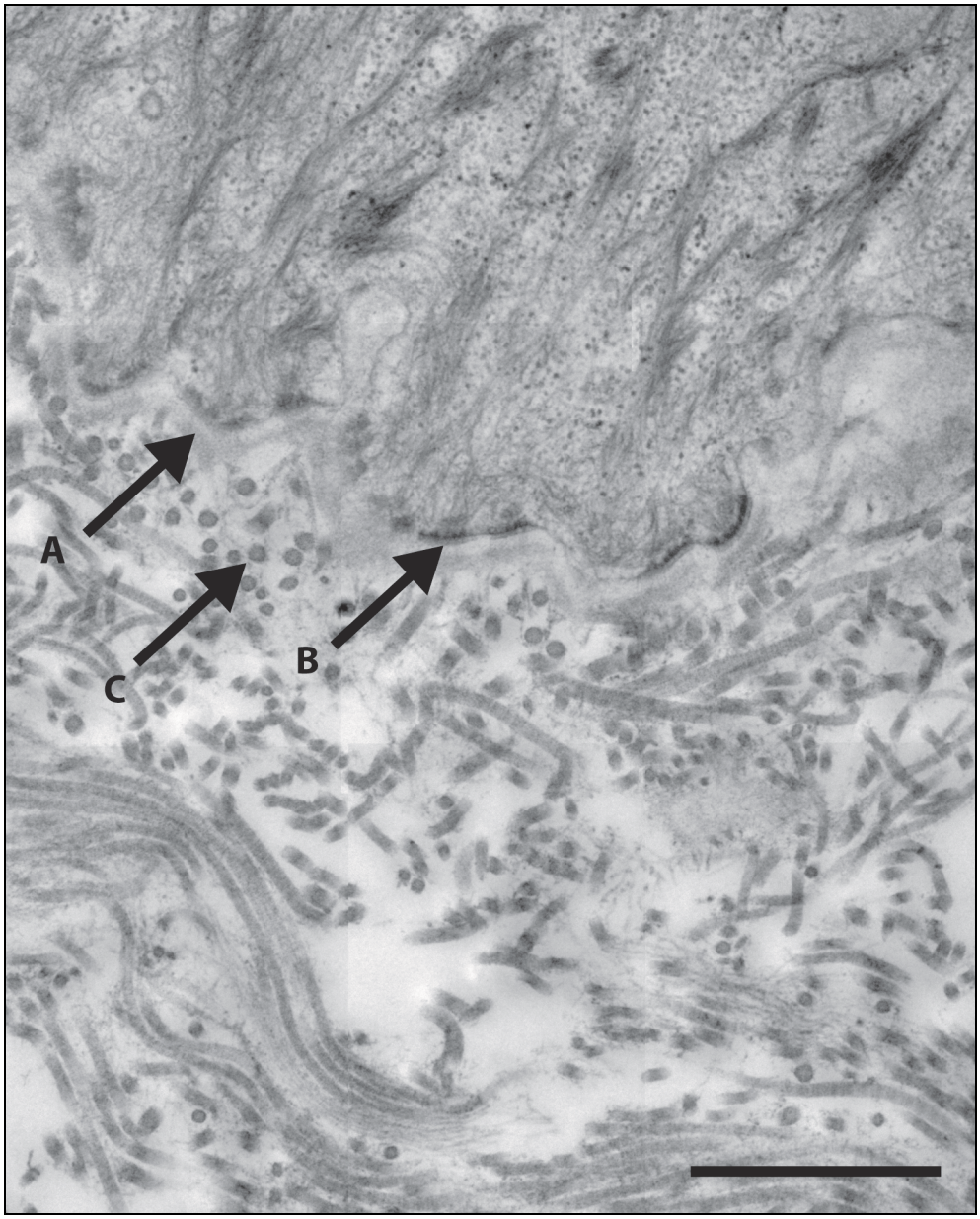

Figure 7 | Ultrastructure of human fibroblast-derived matrix (FDM) based human skin equivalents (HSEs) assessed by transmission electron microscopy. Human keratinocytes were cultured for 13 weeks at the air–liquid interface on FDMs. An overview is shown of the presence of the epidermal and dermal compartment in an FDM-based HSE, containing dermal–epidermal junctions with lamina lucida, laminin densa (A) and regular hemidesmosomes (B). The uniform collagen fibrils indicate their normal processing and proper assembly (C). Scale bar: 1 µm.

irrespective of the culture period, laminin 332 and collagen types IV (data not shown) and VII were all confined to the epidermal–dermal junction, similar to native tissue (Figure 8) and rat-tail collagen-based HSEs.[14] In FDM-based HSEs cultured for 4 weeks similar results were observed (data not shown).

NMF concentration of fibroblast-derived matrix-based HSEs and rat-tail collagen-based HSEs

After evaluation of morphology, proliferation and differentiation status and basement membrane composition in short- and longterm FDM-based HSEs, the concentration of the molecular constituent natural moisturizing factor (NMF), that is essential in skin barrier function, was measured in the stratum corneum of these models in comparison to rat-tail collagen-based HSEs and normal skin. Through this measurement, we assessed the skin barrier function of FDM-based and rat-tail collagen-based HSEs, which depends on proper moisturization, lipid balance, and the presence of humectants, which are the main constituents of NMF. Using confocal Raman spectroscopy the molecular concentration profile for this component was measured in FDM-based HSEs, rattail collagen-based HSEs and normal skin. In normal skin, the NMF reached a relative concentration of 1.2 normalized to keratin, while this constituent was virtually absent in rat-tail collagen-based HSEs (Figure 9). FDM-based HSEs contained an NMF concentration that was 2.5 times lower than in normal skin, but that widely exceeded that of rat-tail collagen-based HSEs (Figure 9).

Discussion

Reconstructed HSEs are widely used by scientists and industry for research purposes and for clinical applications in regenerative medicine. The epithelial–mesenchymal interactions in these skin equivalents are of crucial importance to the quality and functional reliability of the reconstructed epidermis. Therefore, a number of studies focused on the generation and optimization of new threedimensional cell culture matrices to establish better representation of the geometry, chemistry and signalling environment of the native ECM.[15] For dermal scaffold generation, various cellbased approaches have been used to improve epidermal regeneration.[16-18] The dermal matrix presented by Stark et al. consisted of a scaffold consisting of hyaluronic acid fibers and fibrin gel in which fibroblasts were embedded and on which the seeded keratinocytes formed a full epidermal architecture with normalized differentiation status which lasted for a minimum of 12 weeks.[19] Still, the most general used dermal matrix is the collagen matrix, in which the collagen can be of rat or bovine origin. However, the use of fibroblast-populated collagen matrix for long-term studies is hampered by its limited lifespan due to high

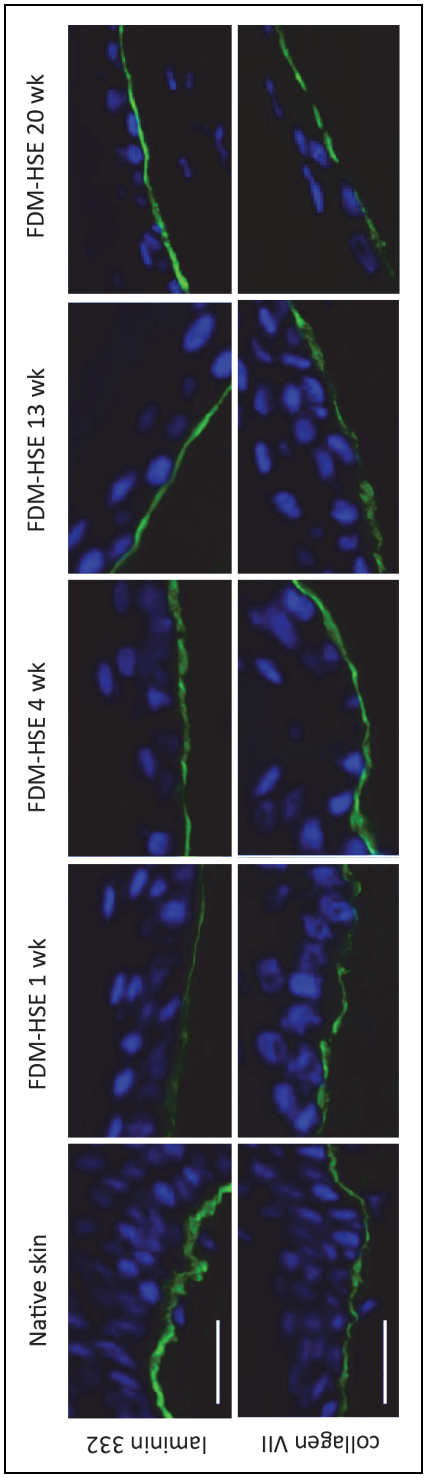

Figure 8 | Detection of basement membrane proteins in human fibroblast-derived matrix (FDM) based human skin equivalents (HSEs) cultured for longer periods. Human keratinocytes were cultured for 1, 4, 13 and 20 weeks at the air–liquid interface on FDMs. Immunofluorescence staining on cross-sections demonstrate that laminin 332 and collagen type VII are expressed at the dermal-epidermal junction, irrespective of the culture period of the FDM-based HSEs. Scale bar: 25 µm.

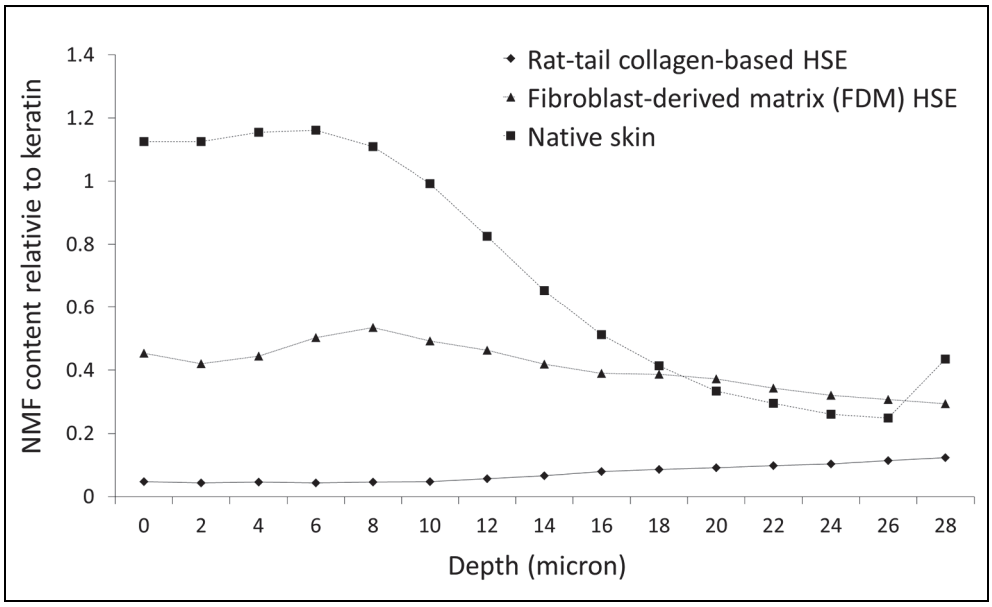

Figure 9 | Semi-quantitative concentration profiles of natural moisturizing factor (NMF) in the stratum corneum of two rat-tail collagen-based human skin equivalents (HSEs) cultured for 8 weeks, two human fibroblast-derived matrix (FDM) based HSEs cultured for 8 weeks, and the forearm of two volunteers (*in vivo* skin) as determined by Raman spectroscopy. NMF was virtually absent in rat-tail collagen-based HSEs (dots), while FDM-based HSEs contained an NMF concentration (triangles) that was 2.5 times lower than in normal skin (squares).

contractibility resulting in poor epidermal homeostasis *in vitro*. The extent of contraction is dependent on the collagen concentration, the number of fibroblasts present in the matrix, and the culture time of the matrices prior to seeding of keratinocytes.[12] To avoid contraction of the dermal compartment, non-contractile matrices can be used such as the natural substrate DED. However, since its uncontrollable variation in thickness, consistent experiments are hard to realize with this substrate. Another impediment is the short lifespan of DED-based skin models. After 8–10 weeks of culture, these skin models have an epidermis harboring only one or two viable cell layers, rendering them unsuitable for long-term studies pertaining to e.g. skin aging and UV carcinogenesis.

In this study we presented a fully human fibroblast-derived matrix (FDM), consisting of human ECM secreted *in vitro* by primary normal human dermal fibroblasts. Using this approach we observed that under specialized circumstances primary human fibroblasts secreted ECM components during the whole culture period with a maximum production of pro-collagen type I in the seventh culture week, being the fourth week after seeding of

normal human epidermal keratinocytes, after which the secretion remains steady up to the eleventh week. This observation indicates that the maximum production of pro-collagen I is linked to maximum cell confluency on the filter insert. An eventual proliferation stop results in stagnation of collagen production starting from week 11. Cross-sections of FDM-based HSEs also revealed epidermal differences when compared to rat-tail collagen-based HSEs. After 2 weeks culture, the epidermis generated on rat-tail collagen-based HSEswas reduced very fast until only 3–4 cell layers were left in the eighth week, while the epidermis generated on FDM-based HSEs maintained its 4–5 viable cell layers up to 13 weeks of culture and slightly decreases to approximately 3 viable cell layers after 20 weeks of culture. These observations emphasize the importance of the microenvironment surrounding the fibroblasts. One could speculate that the keratinocytes cultured on animal-derived collagen may loose their self-renewal capacity due to improper maintenance of the interfollicular stem cell compartment. The proliferative and tissue renewal characteristics of epidermal stem cells (ESCs) of the skin have been clearly demonstrated.[20] Rarely dividing ESC and proliferative progenitor cells play a central role in tissue renewal and repair.[21] Progenitor cells and ESC adhere differentially to ECM components such as collagen type IV and fibronectin, suggesting a stem cells niche with tight adherence to the ECM-rich basement membrane.[22, 23] Among different stem cell markers, cytokeratin 15 (K15) was found to mark putative stem and progenitor cells in the tip of the rete ridges of human skin.[24] Although no rete ridges are present in any of the HSEs presented here, K15 was expressed during the entire culture period irrespective of the dermal matrix used. In another study performed in HSEs, Kaur and her group demonstrated the extensive tissue-regenerative capacity of neonatal human ESCs.[25] However, the questions still remain whether interfollicular ESCs can increase the lifespan of HSEs and why these HSEs lose their self-renewal capacity *in vitro* but not *in vivo* as a graft.[26, 27]

It has been shown that keratinocytes in culture often represent a certain pathologic condition, such as epidermal injury or psoriasis. Under such conditions, the keratinocytes are in an activated state, expressing activation- and hyperproliferation-specific proteins K16 and K17. Having responded to injury, keratinocytes receive a deactivation signal and revert to their normal differentiation program. Based on the results obtained in the present study, it became clear that fibroblasts can produce such signals, since in FDM-based HSEs the expression of K16 and K17 was fully downregulated during the whole culture period up to 20 weeks. In a previous study we demonstrated that a full fibroblast-induced downregulation of K16 expression in keratinocytes occurs on fibroblast-populated collagen matrices already at an earlier time point,[3] indicating that in addition to cell–cell interaction also cell–matrix interaction may be involved in the normalization of epidermal

differentiation *in vitro*. It should be noted that K16 and K17 reappeared at the periphery of FDM-based HSEs (Fig. 5b). These keratins are thought to promote reorganization of the cytoplasmic array of keratin filaments, an event that precedes the onset of keratinocyte migration into the wound site, with K16 induction occurring prior to K17 induction.[28, 29]

As human fibroblasts were directly surrounded by their own secreted microenvironment in FDM-based HSEs, they influenced epidermal proliferation. This growth-promoting effect of fibroblasts is induced by soluble factors released from fibroblasts.[30] In spite of a decreasing proliferation index seen after 6 weeks of FDMbased HSE culture, the localization of the terminal differentiation marker involucrin shifted towards the granulous layer in FDM-based HSEs cultured for 13 weeks or longer. The early differentiation marker K10 was observed in all suprabasal cell layers during the entire culture period, indicating normal differentiation. The microenvironment of FDM-based HSEs did not alter the basement membrane formation as judged by the expression of laminin 332 and collagen types IV and VII when compared to rat-tail collagenbased HSEs.[30] Both proteins were expressed along the epidermal–dermal junction during the entire culture period indicating a proper basement membrane on which keratinocytes can migrate and differentiate. In addition, electron microscopy analyses revealed the presence of essential components of the basement membrane, including dermal–epidermal junctions, hemidesmosomes and anchoring fibers.

It is clear that HSEs generated on different dermal matrices show great similarities to normal human skin. However, one of the key processes impaired under *in vitro* conditions is desquamation as the ultimate representation of terminal differentiation. Desquamation involves the degradation of corneodesmosomes, in which various enzymes participate. Activation of these enzymes is affected by several microenvironmental factors such as pH and water content. The generation of NMF by HSEs is also dependent on the water content in its microenvironment. A water content that is either too low or too high will impair the activity of the enzymes involved in the production of NMF components.[31] A high relative humidity in the microenvironment is, therefore, expected to reduce the formation of NMF, as demonstrated by de Declercq et al.[32] Using Raman spectroscopy we compared both FDM-based HSEs and rat-tail collagen-based HSEs in the presence of NMF.[33] The resulting data indicate that FDM-based HSEs contain higher levels of NMF than the rat-tail collagen-based HSEs, providing grounds to assume that the water and humidity levels are more normalized in FDM-based HSEs than in rat-tail collagen-based HSEs.

The results of the present study indicate that the microenvironment generated by primary human fibroblasts themselves is very suitable to use as a dermal matrix for the generation

of long-lived HSEs. The generated epidermis of resulting HSEs could be maintained up to 20 weeks in culture without the expression of the hyperproliferation markers K16 and K17. In addition, the number of viable cell layers remained constant up to 13 weeks of culture and decreased slightly after 20 weeks of culture. Finally, we have shown that this new generation of HSEs contains a much higher NMF concentration compared to rat-tail collagen-based HSEs. These findings provide a further physiological resemblance to normal human skin and allow for functional studies focused on the effects of long-term exposure to exogenous stressors on epidermal homeostasis and skin cancer.

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