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INVESTIGATIVE REPORTS

Characterization and Comparison of Reconstructed Skin Models: Morphological and Immunohistochemical Evaluation

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Reconstructed human skin equivalents are currently being investigated as *in vitro* models for the prediction of human skin toxicity and irritation responses. Three different industrial reconstructed skin models (EpiDerm, Episkin and SkinEthic) and one in-house equivalent were characterized and compared using light microscopy, immunohistochemistry and reduction of (3-[4,5-dimethylthiazole-2-yl]-2,5-diphenyl tetrazolium bromide) (MTT). Their inter- and intra-batch variation was evaluated. Histological examination showed a completely stratified epithelium in all skin models, which closely resembled normal human epidermis. Low intra-batch variation in tissue architecture was observed in all skin models, but moderate to considerable inter-batch variation was noticed. Evaluation of the expression and localization of a number of differentiation-specific protein markers revealed that all skin models showed an aberrant expression of keratin 6, skin-derived antileukoproteinase, small proline rich proteins, involucrin and transglutaminase. Although variation within batches was low, in particular keratin 6, involucrin and skin-derived antileukoproteinase expression demonstrated some inter-batch variation. Reduction of MTT in vehicle-treated cultures showed high similarities between skin models, but marked differences were observed when 1.0% sodium lauryl sulfate was applied topically for 3 or 16 h. Most pronounced effects were noticed in SkinEthic cultures. Intra-batch variations were low and moderate variations were observed between batches. All skin models tested reproduced many of the characteristics of normal human epidermis and therefore provide a morphologically relevant *in vitro* means to assess skin irritation and other skin-related studies. *Key words: human skin equivalents; keratins; cornified envelope precursors; MTT assay.*

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Product safety testing and risk/benefit evaluation are conducted in order to identify substances which may induce adverse skin reactions and to assess human environmental risks. Although during recent years numerous *in vitro*, non-animal systems have been developed, no international agreement exists for *in vitro* methods as alternatives to animal studies (1, 2). Nevertheless, a promising contribution may be provided by the reconstruction of human skin equivalents *in vitro*.

Nowadays, various types of reconstructed human skin exist. They are generated by growing differentiated keratinocyte cultures on acellular or fibroblast-populated dermal substrates (3–9). These skin equivalents form a multilayered epithelium composed of an organized stratum basale, stratum spinosum, stratum granulosum and a stratum corneum. Furthermore, they display characteristic epidermal ultrastructures (8, 10, 11) and they express markers of epidermal differentiation (6, 12, 13). However, differences between native and reconstructed epidermis are still evident.

Although extensive studies have been performed on the application of skin models as tools for measurement of skin irritation (14–18), no standard guidelines exist with regard to quality control of the models, relevant biological endpoints for assessing skin irritation and their predictive potential for the *in vivo* situation. Moreover, for all applications it is important to know that the results obtained from a skin model are reproducible, both within a given batch and between batches.

The aim of this study was to compare 3 different industrial reconstructed skin models and one in-house equivalent on the basis of light microscopic and immunohistochemical examination, and to evaluate their inter- and intra-batch variation. Moreover, reduction of 3-[4,5-dimethylthiazole-2-yl]-2,5-diphenyl tetrazolium bromide (MTT) was investigated in control cultures and cultures treated with sodium lauryl sulfate (SLS).

MATERIALS AND METHODS

Commercially available skin models

Reconstructed human epidermis. Three human epidermal constructs were used: EpiDerm™ (MatTek Corporation, Ashland, MA, USA), Episkin™ (Episkin SNC, Chaponost, France), and SkinEthic™ (Laboratoire SkinEthic, Nice, France). Two types of EpiDerm were evaluated, i.e. hydrocortisone-free cultures (EPI-200-HCF) and percutaneous absorption cultures (EPI-606A) (7, 19, 20). Episkin skin models consisted cultures grown for 13 days (irritation model) or 20 days (penetration model) at the air-liquid interface (6, 18, 21). SkinEthic cultures were grown for 18 days at the air-liquid interface (8, 22, 23). Upon receipt of the cultures and according to the specifications of the suppliers, Episkin and SkinEthic cultures were stored overnight at 37°C and 5% CO_2, and EpiDerm cultures were stored overnight at 4°C. Three different batches of each model were evaluated, except for 4 batches of EPI-200-HCF cultures and 4 batches of the SkinEthic model. All batches were manufactured between January and July 1998.

In-house skin model. Normal human keratinocytes, cultured as described earlier (24), were seeded on de-epidermized dermis (DED) obtained from human cadaver skin (3). The cultures were first grown under submerged conditions in a mixture of Dulbecco's

modification of Eagle's medium and Ham's F12 medium (3: 1) (ICN Biomedicals Inc., Costa Mesa, CA, USA) supplemented with 5% HyClone serum (HyClone Laboratories Inc., Utah, USA), 100 IU/ml penicillin (ICN), 100 µg/ml streptomycin (ICN), 0.5 µM hydrocortisone (Sigma Chemical Co., St. Louis, USA), 1.0 µM isoproterenol (Sigma) and 0.1 µM insulin (Sigma). After 1 day, the cultures were grown submerged for an additional 2 days in a mixture of DMEM/Ham's F12 medium (3:1) supplemented with 1% serum, 100 IU/ml penicillin, 100 µg/ml streptomycin, 0.5 µM hydrocortisone, 1.0 µM isoproterenol, 0.1 µM insulin, 10 µM carnitine (Sigma), 10 mM serine (Sigma), 1 µM α-tocopherol (Sigma), 5.3×10^{-8} M selenous acid (Johnson Matthey Materials Technology, England), and enriched with a lipid supplement containing 25 µM palmitic acid, 15 µM linoleic acid, 7 µM arachidonic acid and 24 µM bovine serum albumin (Sigma). Thereafter, the cultures were lifted to the air-liquid interface and cultured for an additional 14 days in the same medium, except that serum was omitted, the concentration of linoleic acid was increased to 30 µM and 50 µg/ml ascorbic acid (Sigma) and 1 ng/ml epidermal growth factor (Sigma) were added (11). Culture medium was renewed 3 times a week.

Test agent

SLS (Sigma) was dissolved in distilled water and used at a concentration of 1.0%. After storage overnight upon receipt, fresh medium was added and 26 µl of SLS was applied on the epidermal surface for 3 or 16 h using a filter paper (0.5 cm^2; Finn Chamber), except for Episkin cultures in which 50 µl was applied on a 0.95 cm^2 filter paper.

Morphology

Control and SLS-treated samples were fixed in 4% paraformaldehyde and processed for embedding in paraffin. Vertical sections (5 µm) were cut and stained with haematoxylin and eosin for light microscopic examination.

Antibodies

Antibodies to K1 (LHK1) were kindly provided by Dr I. Leigh (London), K6 antibodies (Ks6KA) were obtained from Progen Biotechnik GmbH (Heidelberg, Germany), and K10 antibodies (DEK10) were obtained from ICN Biomedicals Inc. (Costa Mesa, CA, USA). Antibodies to skin-derived antileukoproteinase (SKALP) were a kind gift of Dr J. Schalkwijk (Nijmegen), transglutaminase I antibodies (BC1) were obtained from Biomedical Technologies Inc. (Boston, MA, USA), involucrin antibodies (SY5) were kindly provided by Dr F. Watt (London), loricrin antibodies (AF62) were obtained from Berkeley Antibody Company BAbCO (Lakeside Dr Richmond, CA, USA), and antibodies to small proline rich proteins (SPRRs) were a gift of Dr D. Hohl (Lausanne).

Immunohistochemistry

Immunohistochemistry was performed on paraffin or cryostat sections. Immunohistochemical analysis of keratin 1 (K1) and transglutaminase was performed on 5 µm cryostat sections. For detection of SKALP, involucrin, loricrin, SPRR2 and 3, a standard method for antigen retrieval on paraffin sections was used. Prior antigen retrieval of K6 and K10 the following modifications were used; for K6, after deparaffinization, sections were immersed in 0.01 M sodium citrate buffer (pH 6.0) for 30 min at 100°C followed by slowly cooling to room temperature over a period of at least 3 h. Incubation with the first antibody was performed overnight. For K10, sections were immersed in preheated (to 100°C) 0.01 M sodium citrate (pH 6.0) for 30 min and were cooled to room temperature in 30 min, followed by digestion in 0.04 mg/ml pepsin in 0.2 M HCl for 15 min at room temperature. After incubation with primary antibodies, the

sections were stained with the avidin-biotin-peroxidase complex method essentially as described by the suppliers (streptABCcomplex/HRP, DAKO, Glostrup, Denmark).

MTT assay

After overnight storage of the cultures according to the specifications of the suppliers, distilled water or 1.0% SLS was applied for 3 or 16 h at 37°C and 5% CO_2. Thereafter, the MTT assay essentially as described by Mosmann (25) was performed. Briefly, after treatment the cultures were rinsed with phosphate-buffered saline and transferred to multiwell plates, each well containing freshly prepared and filtered MTT solution at a concentration of 2 mg/ml culture medium. After incubation for 2 h at 37°C, the cultures were gently blotted on tissue paper and transferred to a multiwell plate containing 3.3 ml isopropanol (acidified with 0.04 N HCl) per cm^2 culture area, and further incubated overnight at room temperature. A 200 µl volume of the extractant solution was transferred to a 96-well microtitre plate for measurement of the optical density at 550 nm and a reference wavelength of 650 nm, using acidified isopropanol as a blank.

RESULTS

Epidermal morphology

Investigation of vertical paraffin sections stained for histology (Fig. 1) revealed a characteristic epidermal stratification in all models. All cell layers were present, including stratum basale, stratum spinosum, stratum granulosum and an anucleated stratum corneum. Differences were observed in the number of living cell layers. EpiDerm cultures (EPI-200-HCF) showed an epithelium consisting of 9–12 living cell layers, whereas the penetration models (EPI-606A) consisted of a lower number of cell layers (5–7). In the latter, the keratinocytes appeared more flattened in the upper cell layers compared with EPI-200-HCF cultures. In particular in lower cell layers in both EpiDerm models, intercellular spaces were clearly visible. The basal cells showed a high cubical shape progressively differentiating into 1–2 layers of flattened granular cells containing keratohyalin granules. Concerning tissue architecture, intra-batch variation was negligible and only very small differences were observed between batches.

The number of living cell layers in Episkin cultures varied between 8–10 in irritation models and 7–8 in penetration models. Remarkably, penetration models consisted of a thick and compact stratum corneum. All Episkin cultures showed 1 or 2 layers of cubically shaped basal cells and a relatively abrupt transition to very flat cells in upper cell layers. Granular cells containing keratohyalin granules were present, but were flat, irregular and compact. Episkin cultures demonstrated only low variation in tissue morphology within batches, but some variation was observed between batches with regard to the number of cell layers and the degree of tissue organization.

SkinEthic cultures demonstrated a lower number of living cell layers (5–7) compared with EpiDerm and Episkin models. A basal layer of perpendicular-oriented, rounded basal cells was shown which flatten out as the apical surface of the tissue is approached. Intra-batch variation was low, but considerable inter-batch was noticed showing variation in thickness of the epidermis and sometimes pycnotic cells were present in the stratum basale.

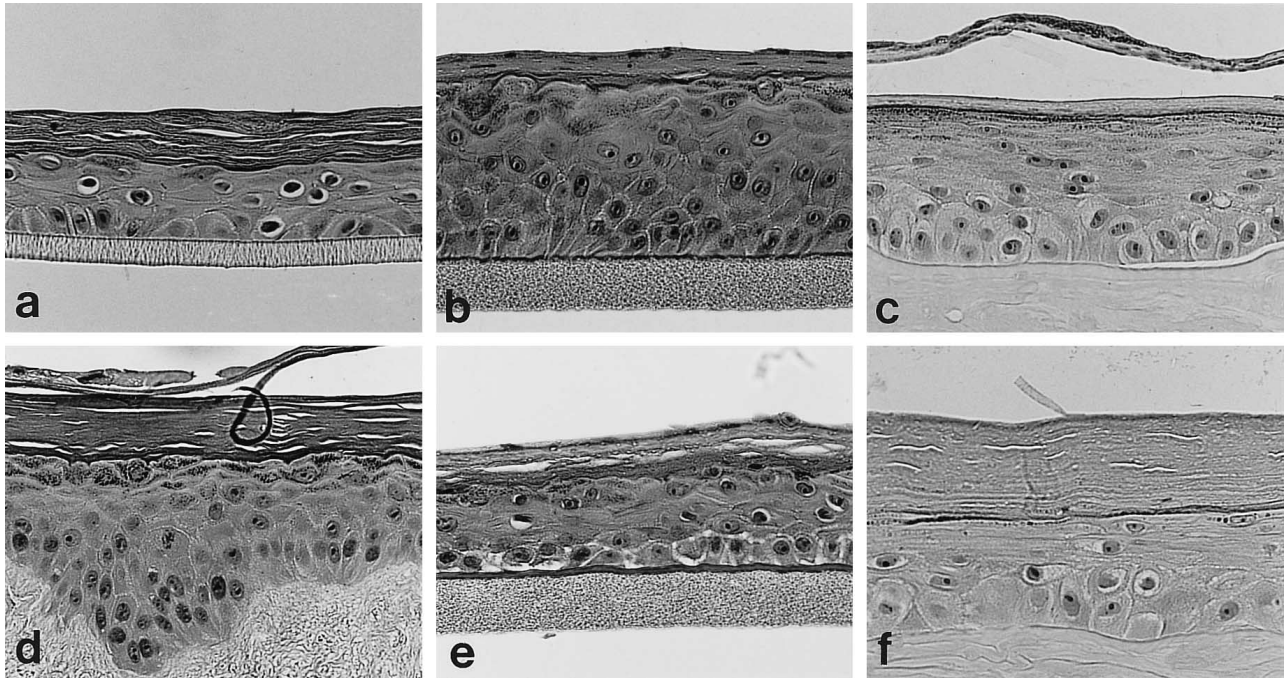The number of cell layers in reconstructed epidermis on de-

Fig. 1. Haematoxylin and eosin-stained paraffin sections of various skin models (*a*) SkinEthic; (*b*) EpiDerm EPI-200-HCF; (*c*) Episkin irritation model; (*d*) reconstructed epidermis on deepidermized dermis (RE-DED); (*e*) EpiDerm penetration model, EPI-606A; (*f*) Episkin penetration model. Each model demonstrated a completely stratified epidermis, the presence of numerous keratohyalin granules and the formation of a coherent stratum corneum. Note the lower number of cell layers in EPI-606A and the thick and compact stratum corneum in Episkin penetration models compared with the irritation models. Representative pictures are shown. All cultures were processed for staining after overnight incubation according to the instructions by the manufacturer.

epidermized dermis (RE-DED) varied between 7–9 showing a regular tissue arrangement, a gradual transition from perpendicular-oriented basal cells to 1 or 2 layers of flattened granular cells expressing keratohyalin granules. Intra- and interbatch variations were low.

Expression and localization of differentiation markers

For examination of the differentiation process, immuno-histochemical staining was performed using several specific keratinocyte differentiation markers, including keratins and precursors of the cornified envelope.

Keratins. As shown in Fig. 2, all skin equivalents expressed K10, which was localized in suprabasal layers similar to its expression in normal epidermis, although in Episkin cultures K10 expression was found in higher epidermal layers (spinous and granular layers) (Fig. 2C, D). Similar observations were made for K1 (data not shown). K6, a protein that is absent in normal human epidermis but is often associated with wound repair, hyperproliferation and abnormal differentiation (26–28), was differently expressed in all models. In EpiDerm cultures (Fig. 2A, B) all suprabasal layers were stained, but expression was more pronounced in lower spinous and granular layers. Surprisingly, K6 was only intermittently expressed in upper spinous layers. Similar observations were made in SkinEthic cultures (Fig. 2E). However, in some SkinEthic cultures K6 was homogeneously expressed in all suprabasal layers. In contrast, in RE-DED cultures K6 expression occurred only in lower suprabasal layers (Fig. 2F). A pronounced K6

staining was shown in all cell layers in Episkin cultures, but some inter-batch variation was evident in staining pattern of the stratum basale showing staining of either all or only some basal cells (Fig. 2C, D). All cultures showed some degree of staining in the stratum corneum. Whether or not this is specific or aspecific staining is not yet known.

Cornified envelope precursor proteins. The expression and localization of cornified envelope precursor proteins; SKALP, SPRR2 and SPRR3, involucrin, loricrin and transglutaminase are shown in Table I. Expression of SKALP, which is not detectable in normal human skin (29), was demonstrated in all skin models. In EPI-200-HCF, SKALP was strongly expressed in spinous and granular layers intracellularly either below the upper cell membrane or throughout the cytoplasm, whilst in EPI-606A expression was much less pronounced and was found only intracellular below the upper cell membrane in the upper stratum spinosum and stratum granulosum. A comparable staining pattern was observed in Episkin and SkinEthic cultures. Although SKALP was more clearly expressed in EPI-200-HCF and RE-DED, very low variation was demonstrated in distribution of SKALP in the epidermal layers both within and between batches. SPRR2 was expressed in granular layers in all skin models similar to normal human epidermis (30). In EpiDerm samples, however, SPRR2 was intermittently expressed in the stratum granulosum and only weak staining was observed in EPI-200-HCF cultures. Although SPRR3 is absent in normal human epidermis, EPI-606A, Episkin irritation models and RE-DED cultures, in EPI-
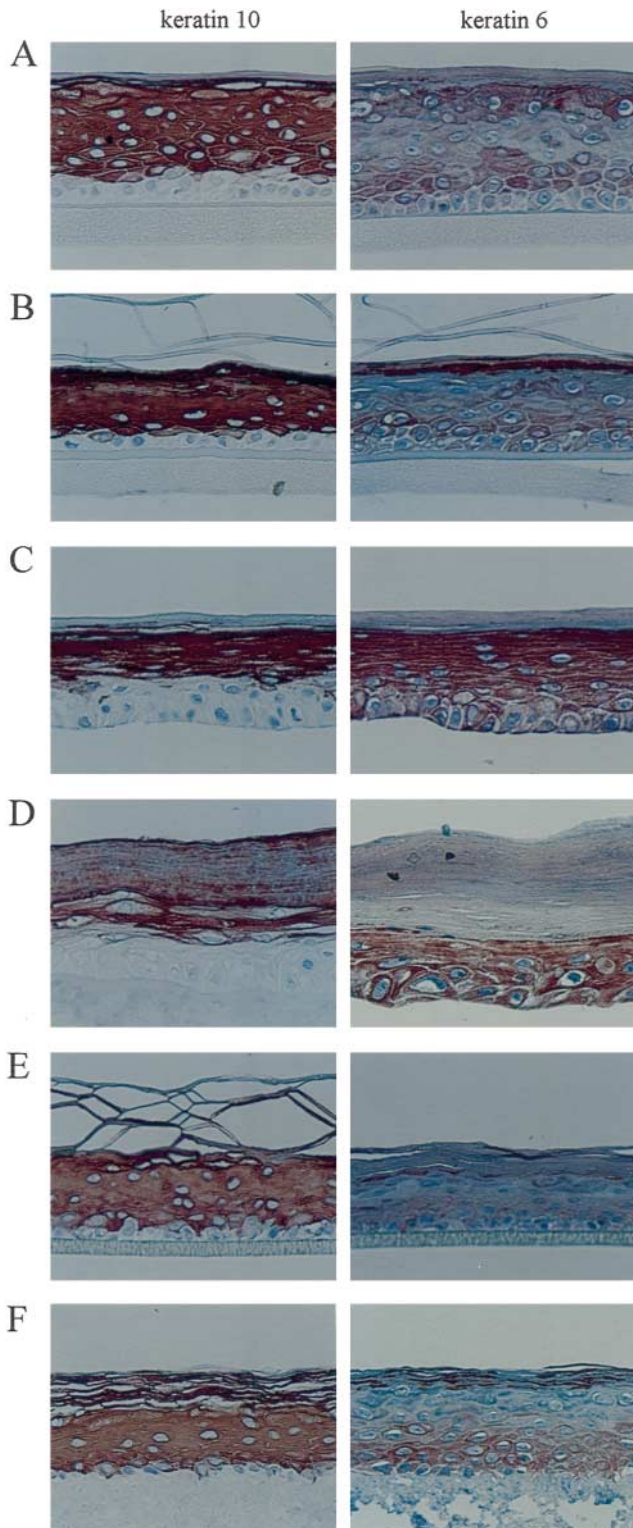
Fig. 2. Immunohistochemical staining of K10 and K6 in vertical paraffin sections of reconstructed skin models (*A*) EpiDerm, EPI-200-HCF; (*B*) Epiderm penetration model, EPI-606A; (*C*) Episkin irritation model; (*D*) Episkin penetration model; (*E*) SkinEthic; (*F*) RE-DED. Note the expression of K6 in all models and the correct expression of K10 in all suprabasal layers, except for Episkin cultures showing staining only in higher suprabasal layers.

200-HCF and Episkin penetration models a moderate intermittent expression was demonstrated, and SkinEthic cultures showed only a slight staining.

Loricrin was expressed in all skin models in the stratum granulosum, similar as in native epidermis (31). Remarkably, in Episkin penetration models expression was absent, except for several cultures that showed some diffuse expression in upper cell layers. Involucrin, normally only present in granular layers (32), was expressed in all suprabasal layers in EpiDerm, SkinEthic and Episkin cultures and in the latter extended even into basal layers. Only RE-DED cultures showed a correct expression of involucrin in the stratum granulosum. Transglutaminase, an enzyme involved in the crosslinking of cornified envelope precursors was prematurely expressed in all skin models. In normal epidermis it is confined to the stratum granulosum (33), whereas in skin models staining was demonstrated in all upper suprabasal layers. Small differences in expression of transglutaminase were observed independent of the model or batch tested.

MTT reduction

To evaluate MTT reduction as a measure of tissue viability and to examine whether qualitative differences in skin equivalents may induce differences in the outcome of the MTT assay, the extent of keratinocyte maturation was varied by exposure of RE-DED cultures to the air for different time intervals.

In cryostat sections of RE-DED specimens harvested at day 3, 6, 14 and 5 weeks after air exposure and incubated for 2 h in 2 mg/ml MTT solution, insoluble blue formazan crystals were present in the lower cell layers whilst no staining was noticed in upper cell layers (Fig. 3). Spectrophotometric determination of the amount of reduced MTT demonstrated no significant differences in optical density in cultures grown at the air-liquid interface for 3, 6 or 14 days or for 5 weeks. In addition, no differences in optical density values were observed between RE-DED cultures grown in the absence or presence of serum (data not shown). Variation in supplementation of the culture medium, like omission of ascorbic acid and α-tocopherol, omission of vitamins and fatty acids during the last 3 days of culture, or variation of fatty acid supplementation did not affect the amount of reduced MTT (data not shown).

Examination of the extent of MTT reduction in different skin models demonstrated high similarities between vehicle-treated conditions, but marked differences were observed when 1.0% SLS was applied topically for 3 or 16 h; conditions at which important changes in histologic appearance of the epidermis have been observed (Fig. 4) RE-DED cultures showed the highest resistance to damaging effects of SLS, resembling the effects of SLS treatment of freshly excised human skin specimens. Although variation in optical density between the industrial skin models was relatively low upon application of 1.0% SLS for 16 h, differences were observed after 3 h application. Most pronounced effects were noticed in SkinEthic cultures, whilst EpiDerm cultures and Episkin cultures showed intermediate optical density. In general, inter- and intra-batch variations were low, implicating high reproducibility of

Table I. *Expression of proteins involved in cornified envelope formation*

Model	SKALP	SPRR2	SPRR3	Loricrin	Involucrin	Transglutaminase
EpiDerm, EPI-200-HCF	SS, SG [strong]	SG [intermittent]	SG [intermittent]	SG	SPB	upper SPB
EpiDerm, EPI-606A	Upper SS, SG	SG [intermittent]	absent	SG	SPB	upper SPB
Episkin, Irritation model	Upper SS, SG	SG	absent	SG	SPB	upper SPB
Episkin, Penetration model	Upper SS, SG	SG	SG [intermittent]	absent	SPB	upper SPB
SkinEthic	Upper SS, SG	SG	SG [weak]	SG	SPB	upper SPB
RE-DED	SS, SG [strong]	SG	absent	SG	SG	upper SPB

SB, stratum basale; SS, stratum spinosum; SG, stratum granulosum; SPB, suprabasal layers.

the MTT assay and low dependence on tissue architecture of the skin equivalents.

DISCUSSION

Our study showed that all reconstructed human skin equivalents evaluated in this study closely resembled native human epidermis. A fully differentiated epidermis was demonstrated with all epidermal strata and a regular tissue arrangement consisting of a physiological number of living cell layers. Both inter- and intra-batch differences were evident with regard to morphological and biochemical parameters.

K1/K10, which are localized in all suprabasal layers in native human skin (34) and thus serve as early markers of the epidermal differentiation process, were shown to be correctly expressed in all models, except for Episkin cultures in which a later onset of expression was demonstrated implicating a delayed epidermal differentiation in these cultures. The proliferation/differentiation balance, however, is disturbed in all skin models as was demonstrated by the presence K6 and SKALP, which are absent in normal epidermis, but which have been demonstrated in hyperproliferative epidermis and during wound healing (27–29, 35). Localization of K6 expression varied between models, which may indicate differences in tissue homeostasis between models. Our results clearly showed that a low SKALP expression did not parallel a decreased K6 staining. In support of these findings, it has been reported that regulation of keratinocyte proliferation can be separated from the regulation of K6 expression (10,

26). In this view, cultures may reveal a normal proliferation rate but nevertheless express K6 and SKALP.

Concerning the other markers of terminal differentiation, some differences in expression were found in the skin models. In particular the presence of SPRR3 in EpiDerm, Episkin penetration and SkinEthic cultures, and the premature expression of transglutaminase and involucrin in all models, support the observations of an imbalance in keratinocyte homeostasis and possible alterations in the synthesis of cornified envelope precursors. The ubiquitous extension of differentiation-specific protein markers in lower epidermal layers has previously been shown to occur in epidermal hyperplasia and during tissue repair (26, 28, 36, 41). In general, differences in the expression of these markers between skin models and the inter- and intra-batch variation may be primarily a result of culture conditions (10, 12, 36–38), implicating that an improvement in culture conditions may be sufficient to achieve a normalized protein distribution.

Besides a balanced proliferation/differentiation status of the epidermis, the development of a proper stratum corneum is equally important as it plays a key role in skin barrier function and therefore in the response to toxic substances. To determine cell survival after a toxic assault, the colorimetric MTT reduction assay that measures the activity of various dehydrogenase enzymes in active mitochondria, has often been used in both monolayer cultures and epidermal equivalents (15, 18, 21, 25, 39, 40). Our data showed that MTT is mainly reduced in the lower cell layers and that optical density in RE-DED cultures was not significantly influenced by variation in epidermal thickness, suggesting that
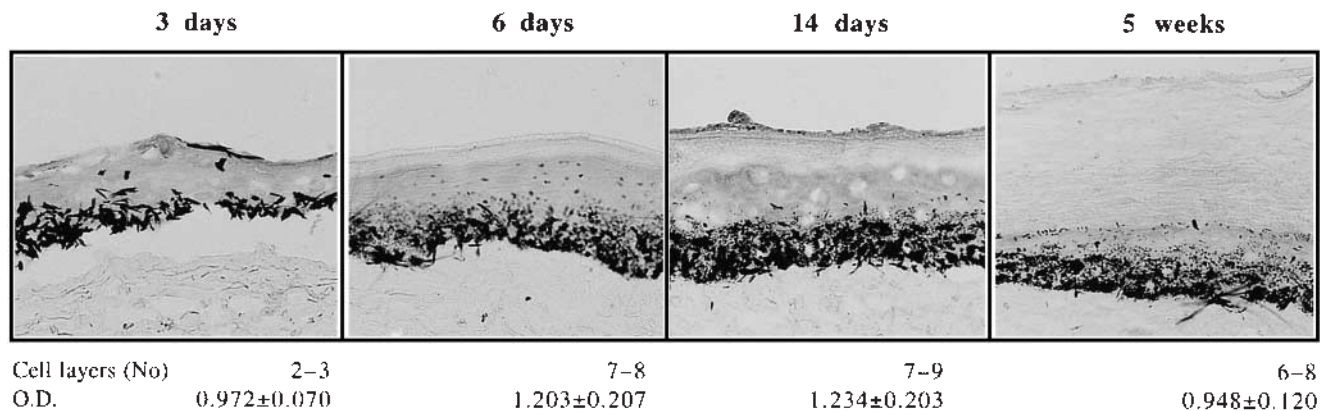

	3 days	6 days	14 days	5 weeks
Cell layers (No)	2–3	7–8	7–9	6–8
O.D.	0.972±0.070	1.203±0.207	1.234±0.203	0.948±0.120

Fig. 3. Nuclear fast red staining of RE-DED cultured for 3–5 weeks at the air-liquid. Tissue specimens were frozen in liquid nitrogen and embedded in TissueTek after MTT reduction for 2 h at 37°C. Data are presented as the mean optical density (OD) ±SEM of triplicate measurements in two independent experiments. Values are given per 0.8 cm^2 culture area per 2.6 ml extraction solution.
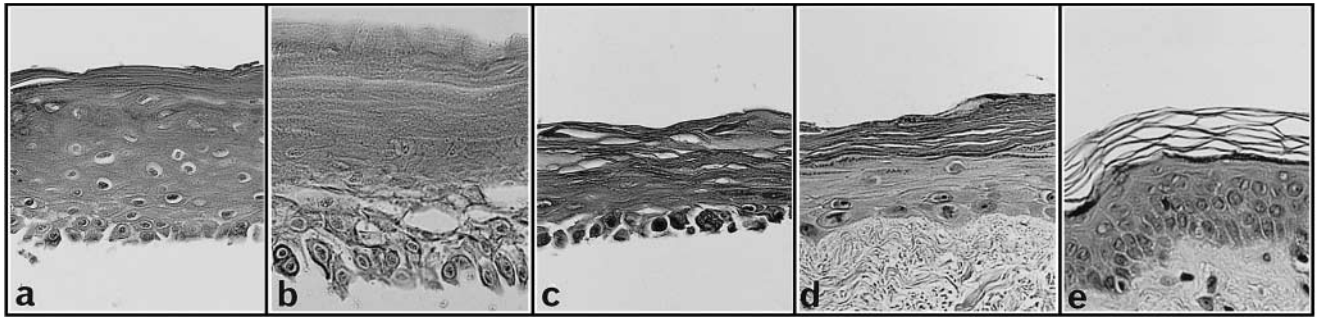
Cell layers (No)	9–12	8–10	5–7	7–9	6–10
Culture area (cm^2)	0.6	1.1*	0.6	0.8	0.8
Extration (ml)	2.0	2.6	2.0	2.6	2.6
O.D. 1					
3 h	1.181±0.082	1.161±0.178	0.815±0.095	1.008±0.018	n.d.
16h	0.980±0.064	0.927±0.088	0.741±0.033	0.869±0.011	0.721±0.075
O.D. 2					
3 h	0.573±0.269	0.149±0.046	0.063±0.045	0.988±0.052	n.d.
16 h	0.068±0.006	0.043±0.009	0.008±0.005	0.857±0.091	0.830±0.098

Fig. 4. Haematoxylin and eosin stained sections of reconstructed skin models treated with 1.0% SLS for 3 h. (*a*) Epiderm, EPI-200-HCF; (*b*) Episkin (irritation model); (*c*) SkinEthic; (*d*) RE-DED; (*e*) Excised skin. MTT reduction as a measure of viability in different skin models. Cultures treated with vehicle (O.D. 1) or with sodium lauryl sulfate (O.D. 2) for 3 h were compared with treatment for 16 h. (*) Circular biopsies (0.8 cm^2) were taken before extraction; n.d. = not done. Data are presented as the mean optical density (OD)±SEM of triplicate measurements in 3 different batches.

terminally differentiated keratinocytes may not be involved in MTT reduction although these keratinocytes are still metabolically active. In addition, determination of MTT reduction did not demonstrate significant differences between the various skin equivalents in the vehicle-treated cultures. However, the optical density measured after application of vehicle for 16 h tended to be lower compared with 3 h treatment with the vehicle, indicating a slightly impaired barrier function. SLS treatment resulted in optical densities close to zero in the industrial skin models, i.e. no MTT reduction occurred due to cell death. Differences in susceptibility to SLS between the industrial skin models were only evident at early time points after topical application of the irritant and indicated that EpiDerm cultures were most resistant to SLS-induced toxicity. In contrast, in excised skin and in RE-DED cultures only a slight decrease in mitochondrial metabolic activity was noticed, clearly indicating that measurement of MTT reduction indirectly reflects the quality of the stratum corneum barrier and that the MTT assay may be used to assess both the ability of test compounds to penetrate the stratum corneum as well as their ability to affect cellular viability.

In conclusion, although substantial progress has been made in improvement of the quality of skin equivalents with respect to tissue architecture and function, their phenotype still represents that of a hyperproliferative tissue or of a recently healed wound. Furthermore, the quality of commercially available skin equivalents depends not only on culture conditions but also on package and shipping procedures. Both aspects should be considered when aiming for further progress of the quality of *in vitro* reconstructed human skin.

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