Epidermal Homeostasis in Long-Term Scaffold-Enforced Skin Equivalents

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Epidermal homeostasis is understood as the maintenance of epidermal tissue structure and function by a fine tuned regulatory mechanism balancing proliferation and cell loss by desquamation and apoptosis. The lack of appropriate experimental models has largely prevented a better understanding of the regulatory mechanisms controlling epidermal tissue homeostasis in human skin. Keratinocyte culture studies had revealed a strict dependency of regular epidermal differentiation on dermal interactions only accomplishable in three-dimensional skin models. As major drawbacks, conventional models, employing collagen hydrogels as dermal equivalents (DEs) exhibit a rather poor stability and limited lifespan. Here, we present an improved stabilized *in vitro*-model for long-term growth and differentiation of keratinocytes providing the basis for tissue homeostasis. Keratinocytes were grown on DEs reinforced by modified hyaluronic acid fibers (Hyalograft-3D) and colonized with skin fibroblasts, producing genuine dermis-type matrix. These skin equivalents (SEs) develop superior epidermal architecture with regular differentiation and ultrastructure. Critical aspects of differentiation, still unbalanced in early stages, are renormalized, most strikingly the coexpression of keratins K1/K10, downregulation of regeneration-associated keratins (K16), and restriction of K15 to the basal layer. The strict localization of integrins to basal cells underlining restored tissue polarity, the drop of keratinocyte growth rates towards physiological levels and the rapid formation of a mature basement membrane with abundant anchoring fibrils are altogether features fulfilling the criteria of tissue homeostasis. Therefore, these scaffold-based SEs not only allow for studying homeostasis control but also for the first time provide proper experimental conditions for establishing a stem cell niche *in vitro*.

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INTRODUCTION

Epidermal homeostasis has been defined as the maintenance of tissue integrity by sustaining a constant epidermal cell pool balancing the rate of mitosis and the rate of cell loss by desquamation and apoptosis (Marks, 1980; Budtz, 1986). In a broader sense, epidermal homeostasis is understood as the maintenance of epidermal tissue structure and function. In skin, epidermis is undergoing a perpetual complex differentiation process, which, however, is not strictly autonomous, but also linked to the underlying connective tissue (Fusenig, 1994). The achievement and maintenance of the equilibrium between cell proliferation and cell loss is precisely controlled in the healthy state. To preserve this tissue homeostasis, an array of regulatory mechanisms is exerted by epithelial–mesenchymal interactions, which is to a large extent mediated by diffusible factors (Werner *et al.*, 1992; Fusenig, 1994; Maas-Szabowski *et al.*, 1999, 2000). However, further important factors involved in the regulation of epidermal homeostasis are the composition and structural organization of the extracellular matrix (ECM) the epidermis is confronted with, in particular, the epidermal basement membrane (BM) (Fuchs *et al.*, 1997; Watt, 2002; Breitkreutz *et al.*, 2004). With regard to tissue integrity, these structural components have apparently a permissive and a protective function, but seem to be also actively involved in mediating or coregulating external signals for the epidermis (Pujuguet *et al.*, 2000).

The investigation of these interactions in the organism is largely hampered by the complexity of the *in vivo* situation. Moreover, as far as human skin is concerned, the lack of appropriate experimental models has largely prevented a better understanding of the regulatory mechanisms maintaining and controlling epidermal tissue homeostasis. In order to address these problems, three-dimensional organotypic

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Abbreviations: BM, basement membrane; 3D, three-dimensional; DE, dermal equivalent; ECM, extracellular matrix; sc-DE, scanfold-reinforced DE; SE, skin equivalent

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cocultures have been developed as *in vitro* skin equivalents (SEs) to represent the major principles of skin biology, consisting of epidermal keratinocytes growing on dermal equivalents (DEs) comprising collagen gels with incorporated fibroblasts (Bell *et al.*, 1981; Mackenzie and Fusenig, 1983; Boukamp *et al.*, 1990; Parenteau *et al.*, 1991; Mackenzie *et al.*, 1993). Although representing strongly simplified culture models, those SEs permit a far-reaching recapitulation of the keratinocyte differentiation program *in vivo*, and have, therefore, been exploited successfully as experimental tools to the better understanding of epidermis biology. This includes, for example, the in-depth characterization of mutual paracrine mechanisms in epithelial–mesenchymal interactions controlling epidermal regeneration (Smola *et al.*, 1993; Maas-Szabowski *et al.*, 2000, 2001; Szabowski *et al.*, 2000) (see Figure 1), the regulation of BM formation (Fleischmajer *et al.*, 1998; Smola *et al.*, 1998; Breitkreutz *et al.*, 2004) the role of fibroblasts in epidermal regeneration (Contard *et al.*, 1993; el Ghalbzouri *et al.*, 2002, 2004), and the development of the *stratum corneum* barrier (Ponec *et al.*, 1997).

As major drawbacks of these SE models, some overt deficiencies such as shrinkage and restricted lifetime limited their use in elucidating the mechanisms regulating balanced growth and differentiation in a stabilized tissue, that is, skin homeostasis, and indicated the need for further optimization of those SEs. Obviously, particular improvements are required in regard to the ECM composition and physical stability of the dermal compartments. Previous reports on scaffold-reinforced DEs (sc-DEs) (Yannas *et al.*, 1989; Cooper *et al.*, 1991; Fleischmajer *et al.*, 1991; Berthod *et al.*, 1996) prompted us to develop a novel type of DE that combines several advantageous features. First, a biocompatible scaffold material confers an appropriate geometric configuration for dermal tissue formation as well as the required mechanical stability without restraining analytical processing such as suitability for cryotomy. Second, the use of fibrin gels improves the colonization of the scaffold by fibroblasts, provides a provisional matrix well suited for keratinocyte growth (Pellegrini *et al.*, 1999; Ronfard *et al.*, 2000), and reduces the precultivation period before keratinocyte seeding. Third, a natural ECM is produced and assembled by skin fibroblasts in the scaffold yielding an authentic dermis-type matrix.

The scaffold utilized herein is a nonwoven material consisting of esterified hyaluronic acid fibers, which is certified for medical use (Hyalograft-3D, provided by Fidia Advanced Biopolymers). Being biodegradable and nonimmunogenic, such materials have been experimentally and clinically applied for tissue engineering of cartilage and skin as well as dermal regeneration (Aigner *et al.*, 1998; Campoccia *et al.*, 1998; Zacchi *et al.*, 1998; Galassi *et al.*, 2000). We had selected this special modification because of its uniform geometry and its well-suited mechanical, biochemical, as well as its genuine cell growth promoting properties.

The objective of this study was to create a stable *in vitro* model for long-term culture enabling mechanistic studies on skin regeneration and homeostasis. This was achieved by elaborating an optimized protocol for tissue engineering of SEs based on that novel type of sc-DEs. A comprehensive characterization is presented revealing that during considerably prolonged cultivation periods epidermal proliferation and differentiation normalized, reaching a state of tissue homeostasis, and that this was a consequence of an *in vivo*-like maturation of the ''dermal'' matrix, involving also regular BM structures. These tissue engineered SEs represent a new generation of *in vitro* skin models with advanced renormalization in both the epidermal and the dermal compartment. Thus, from their consequent application further advancements can be expected in basic research in skin biology in particular in the regulation of tissue homeostasis, but also in applied science fields such as *in vitro* pharmaco-toxicology testing and in the development of skin substitutes for clinical use.

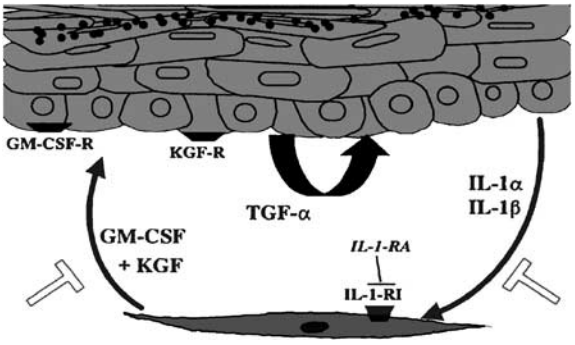

Figure 1. Schematic representation of autocrine and paracrine regulatory circuits engaged in epithelial–mesenchymal interactions controlling growth and differentiation in SEs. Upon skin injury, keratinocytes release IL-1 that enhances expression and release of FGF-7/KGF and GM-CSF, potent stimulators of keratinocyte proliferation. In addition, transforming growth factor-α is acting as an autocrine keratinocyte growth factor (taken from Maas-Szabowski *et al.*, 2001, with permission of *Journal of Investigative Dermatology*).

RESULTS

Production of scaffold-based DEs

So far, using collagen hydrogels as matrix for DEs in constructing skin equivalents in culture (col-SEs), we have encountered certain deficiencies such as poor stability, incomplete differentiation, and early degeneration. Since maintenance of integrity of the SEs apparently was a limiting factor, we aimed to stabilize DEs by using a nonwoven fibrous scaffold based on esterified hyaluronic acid (Hyalograft-3D), a biocompatible, biodegradable, and nonimmunogenic material (Campoccia *et al.*, 1998) (Figure 2). The sc-DEs were constructed by inserting skin fibroblasts, which then synthesized and filled the 3D scaffold with native ECM.

In initial attempts, fibroblasts were inoculated simply as cell suspensions with the result of extreme variations in numbers of cells that were trapped in the scaffold and started growing. Therefore, we elaborated a more standardized seeding technique for improved scaffold colonization by exploiting Tisseel/Tissucol®, a two-component fibrin glue

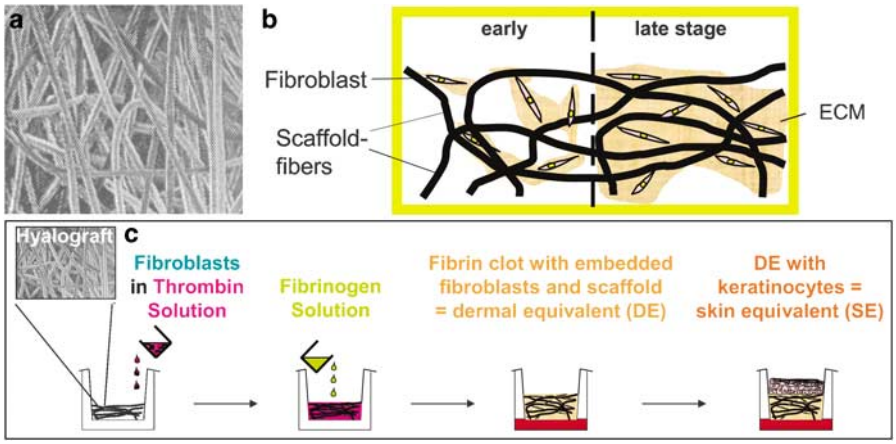

Figure 2. **Concept of scaffold-based DEs.** (**a**) Scanning electron microscopy of Hyalograft 3D-scaffold showing its 3D fibrous organization, original magnification × 80. (**b**) Schematic view of sc-DEs with accumulation of authentic fibroblast matrix. (**c**) Flow-chart of the production of DEs by seeding fibroblasts suspended in fibrin gel solution into the scaffolds.

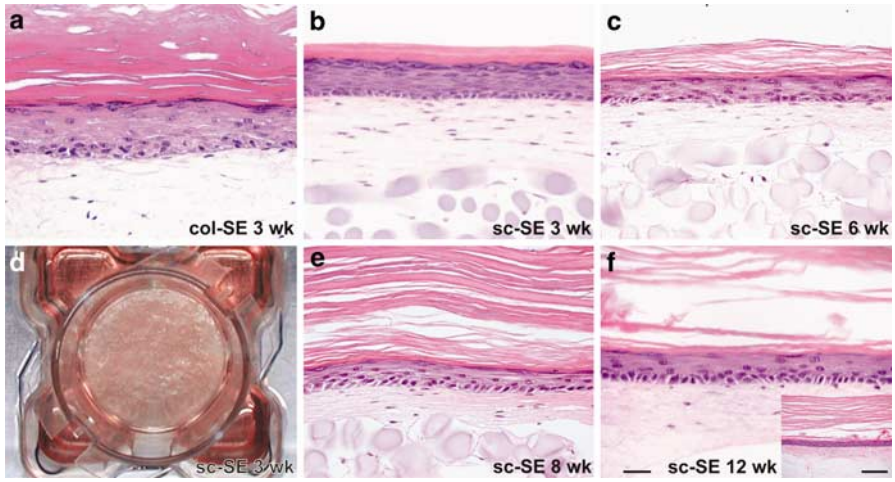

Figure 3. **Histologic and macroscopic appearance of long-term SEs.** (**a**) A 3-week-old collagen gel-based organotypic coculture (col-SE) is compared to sc-SE at (**b**) 3, (**c**) 6, (**e**) 8, and (**f**) 12 weeks. Maintenance of viable cell layers up to 12 weeks with increased thickness of *stratum corneum*. (**d**) Neither distortion nor shrinkage of the sc-SEs was observed over the entire cultivation period, here shown at 3 weeks. (**f**, inset) The entire thickness of the *stratum corneum* is shown at lower magnification. Bars: 50 μm and 250 μm in inset.

preparation. The fibroblasts applied in a diluted fibrinogen/thrombin mixture were detained in the interfilament space of the scaffold at a defined cell density after clot formation. The number of fibroblasts steadily increased during subsequent cultivation. Since enzymatic release of fibroblasts was never complete and yielded inconsistent results, their number was determined by measuring the level of DNA in total extracts. The evenly dispersed fibroblasts actively produced matrix components that rapidly replaced the fibrin gel. With culture time, the fibroblast derived ECM accumulated in organized deposits initially comprised mainly of fibronectin and tenascin (see Figure 5) simulating provisional matrix, followed by type I and type III collagen as well as perlecan/heparan sulfate proteoglycan and other components of the dermal ECM as shown by immunostaining and Western blot (not shown here, but see Stark *et al.*, 2004).

Impact of DEs on epidermal histogenesis and differentiation

The regeneration of a regular epidermis occurred much faster and was better organized in SEs on scaffold enforced genuine dermal equivalents (sc-SEs) as compared to those on collagen gels when analyzed in 2- and 3-week-old cultures (Figure 3). Thus, already in histologic sections a more regular arrangement and polarization of cuboidal basal keratinocytes (*stratum basale*) was observed, indicating an increased stability of epidermal architecture. The more prominent keratohyalin granules in the uppermost viable layers (*stratum* stratum granulosum) suggested an enhancement of late differentiation processes. As direct consequence of the mechanical reinforcement in the sc-DE, the shrinkage of the cocultures, commonly encountered with collagen gels, was completely prevented as obvious by the lack of typical wrinkles in the *stratum corneum*.

Whereas epidermal structures were usually degenerated in col-SE cultures older than 3 weeks, keratinocytes continued to proliferate and differentiate for at least 12 weeks on sc-DEs (Figure 3). This was among others indicated by the thickness of the *stratum corneum* that steadily increased with time in culture exhibiting only ''artificial desquamation'' during histology processing. Along with the formation of an orthokeratinized *stratum corneum* typical differentiation markers were expressed. The antibody against the differentiation-specific keratin K10 also labeled the total *stratum corneum* and clearly visualized its increasing thickness with time in culture (Figure 4a–d). Similarly, all other keratinization markers were typically expressed and localized such as filaggrin, tissue-transglutaminase, repetin, and keratin 2e (not shown here, but see Stark *et al.*, 2004). A significant feature of differentiating keratinocytes is the cornified envelope component loricrin, a late marker of the differentiation process (Steinert, 2000). In contrast to involucrin that exhibited an identical suprabasal localization in epidermal tissue of sc-SEs and col-SEs (Stark *et al.*, 2004) loricrin was present in higher amounts and continuously distributed in the distal portion (upper spinous and granular layer) of the epidermis developed in sc-SEs (see Figure 7).

A very important feature of normal epidermal keratinization is the formation of a barrier function as indicated by the production of tight junctions in the upper *stratum granulosum* (Schluter *et al.*, 2004). This is visualized by labeling the typical components of tight junctions such as occludin, ZO-1, and claudin-1, here shown for occludin that is typically localized in SEs on sc-DEs throughout a culture period of 12 weeks (Figure 4e–h).

Likewise, no substantial differences between the epidermis formed on sc-SEs and in skin could be identified for junctional proteins involved in cell–cell adhesion such as E-cadherin or the desmosomal components plakoglobin and desmocollin-1, expressed suprabasally (not shown here, but see Stark *et al.*, 2004).

Different from that, striking changes in the localization of epidermal differentiation markers became evident when individual keratin expression patterns were analyzed in sc-SEs during long-term culture. This said, K1 and K10 proved to be sensitive indicators for the onset of the tightly controlled differentiation program, that is, tissue homeostasis. K1 and K10 are normally expressed very early in the process of epidermal differentiation, although their expression pattern differs with K10 being present in all suprabasal layers, while K1 expression only starts later (second suprabasal layer). We previously showed that this uncoupled expression of K1 and K10 coincided with epidermal hyperplasia and was maintained in organotypic cocultures of col-SEs throughout the 3 weeks cultivation time (Figure 5) as well as in early (1 week) transplants indicating lack of epidermal tissue homeostasis (Stark *et al.*, 1999). On the other hand, when double stained with monospecific K1 and K10 antisera, epidermis reconstructed on sc-DEs clearly demonstrated restoration of coexpression of K1 and K10 with culture time as seen in native epidermis (Figure 5).

K15 proved to be another sensitive indicator for the functional status of homeostasis in epidermis. K15 belongs to the cytokeratin complement of stratified epithelia, which *in vivo* is generally localized in the basal layer (Figure 5) (Waseem *et al.*, 1999; Werner *et al.*, 2000). An expanded epithelial localization of K15 is linked with deregulated growth of squamous epithelia in hyperplastic or pathological states (Leube and Rustad, 1991). Accordingly, K15 was detected it in a broadened zone largely exceeding the basal layer in 3-week-old col-SEs (Stark *et al.*, 2004) and even so in 3-week-old sc-SEs. However, at later stages in sc-SEs, K15 was largely confined to basal cells as expected from intact skin (Figure 5).

In agreement with an extended expression of K15 also cytokeratins K6, K16 and K17 are expressed during regenerative conditions in skin such as wound re-epithelialization while they are absent from normal homeostatic interfollicular

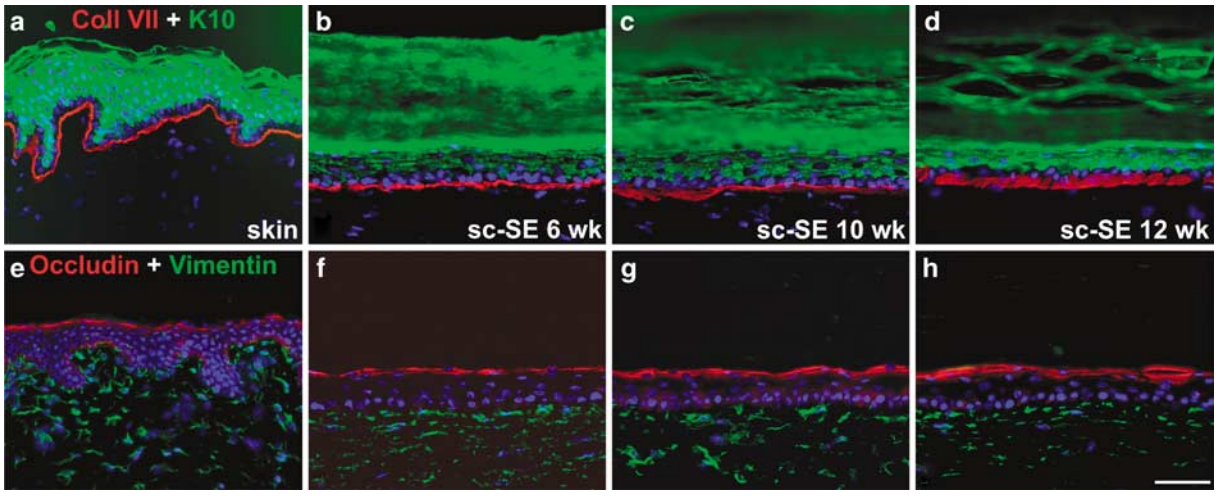

Figure 4. **Regular expression of structural proteins indicative for advanced epidermal histogenesis.** (**a–d**) K10 and type VII collagen in skin and sc-SE at 6, 10, and 12 weeks. (**e–h**) The tight junction component occludin reveals a regular distribution in the epidermal *stratum granulosum*; in parallel, the fibroblast population in the DEs demarcated by vimentin stays fairly constant. Bar: 50 μm.

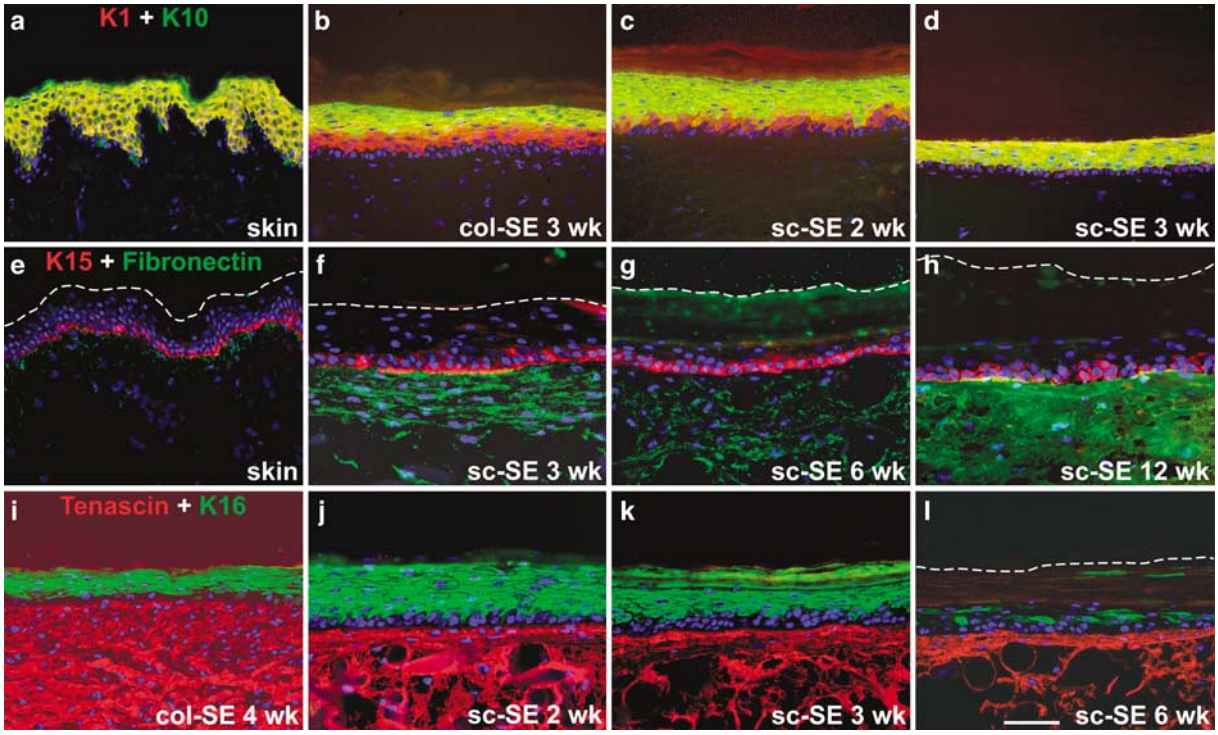

Figure 5. **Expression patterns of indicators for renormalization of epidermal differentiation.** (**a**–**d**) The coexpression of K1 and K10, typical for native epidermis (**a**), is decoupled *in vitro* and stays dissociated on collagen in col-SEs (**b**), whereas it is progressively restored on authentic matrix in sc-DEs (at (**c**) 2 and at 3 weeks (**d**)). K15, specifically located in the basal layer of native epidermis (**e**), resumes this distribution in the epidermis of scaffold based SE (**f**–**h**). The hyperplasia-associated K16, generally expressed *in vitro* and in cocultures on collagen gels, (**i**) is significantly reduced with time on sc-SEs (**j**–**l**). The provisional ECM-component tenascin also shows a time dependent decrease. The surface of the *stratum corneum* is demarcated by dashed lines. Bar: 50 μm.

epidermis. In cultured keratinocytes, they are generally expressed and, due to their stability and slow intracellular turnover, they are often maintained beyond their actual synthesis (Breitkreutz *et al.*, 1993) as also demonstrated for transplants of keratinocytes (Boukamp *et al.*, 1990). Thus, in accordance with earlier findings (Stark *et al.*, 1999), high amounts of K16 remained detectable suprabasally in conventional col-SEs throughout the entire culture period (Figure 5i) and was present in 2-week-old sc-SEs (Figure 5j). Thereafter, K16 expression was reduced in sc-SEs cultures, showing only staining in the spinous and granular layers, and at 6 weeks only some remnant patches in the upper layers (Figure 5k and l). Altogether, the normalization of these markers are interpreted as strong indicators for a normalized and consolidated epidermal differentiation, that is, tissue homeostasis, revealing a nearly physiological regulation under the influence of authentic matrix in sc-SEs.

Modulation of keratinocyte proliferation with normalized differentiation

Another sensitive marker for the transition from wound regeneration to tissue homeostasis is the normalization, that is, reduction of cell proliferation (Breitkreutz *et al.*, 1997). When analyzing keratinocyte proliferation in sc-SEs by BrdU-labeling and staining of the labeled cells by immunofluorescence, the number of proliferating cells – being exclusively situated in the *stratum basale* – continuously decreased during the first 6 weeks reaching levels of 5–10% (Figure 6). Considering a hypothetical length of S phase of 6–8 hours, and a labeling time by BrdU of 18 hours, a ''mean proliferation index'' of 2–5% of basal cells can be estimated. This physiologic rate of epidermal proliferation, as also estimated for normal human epidermis *in situ,* is comparable to that found in late-stage transplants of keratinocyte cultures and in skin explants *in vitro* (Breitkreutz *et al.*, 1997; Stark, unpublished observations).

Ordered deposition of BM components and polarized integrin expression

Essential for epidermal and thus, skin integrity is the correct expression and structural assembly of BM constituents. Generally, the majority of these components was already found in SEs on collagen gels, such as laminin-5, laminin-1/10, type IV collagen, nidogen, and perlecan/heparansulfate proteoglycan (Stark *et al.*, 2004). However, in sc-SEs the deposition of the different components was more polarized and confined to the epidermal–dermal border zone particularly at culture stages longer than 3 weeks (see Figure 6). Moreover, the amount of type VII collagen deposited in the BM zone was clearly enhanced in sc-SEs starting already at 3 weeks of culture, and further increased at later stages, indicating a more regular and advanced BM maturation (see Figure 4).

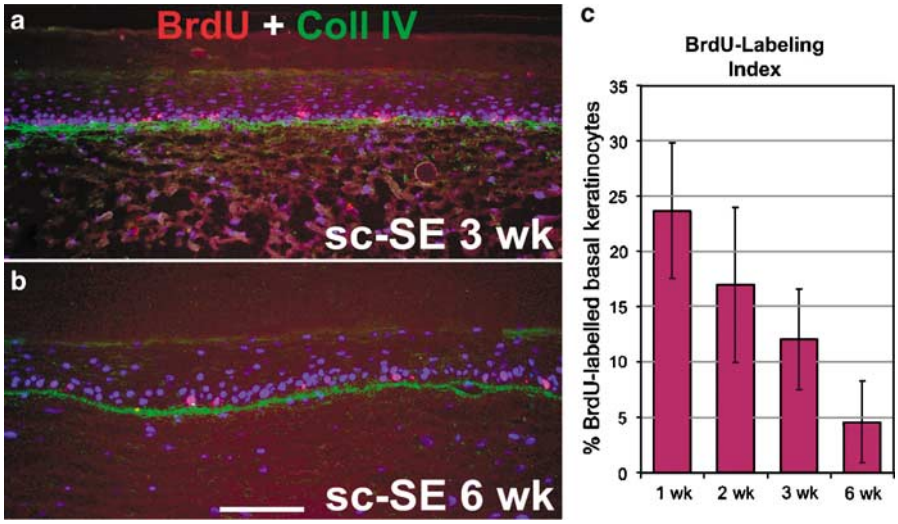

Figure 6. **Normalization of keratinocyte proliferation.** (**a, b**) Basal localization of proliferating keratinocytes and (**c**) decreasing kinetics of epidermal proliferation in scaffold-based skin equivalents as determined by BrdU incorporation. Labeling indices were calculated as ratio of BrdU positve to total number of basal keratinocyte nuclei. The combined data of two representative experiments are shown and expressed as mean percentage values ± SD. Bar: 100 μm.

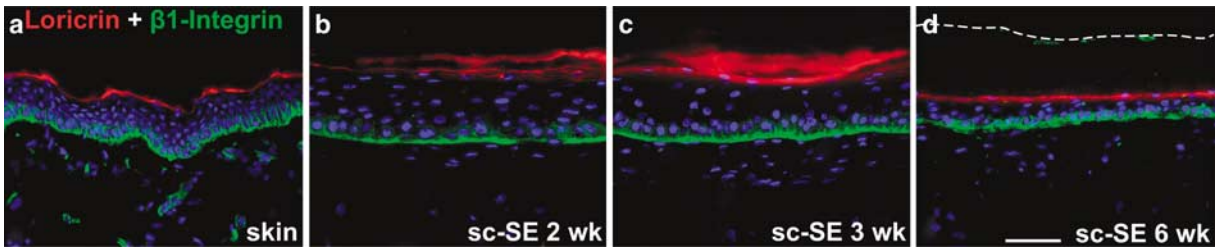

Figure 7. **Distribution of β1-integrins in sc-SEs.** (**a**) Regular pattern of localization of β1-integrin as in native epidermis suggesting (**b–d**) renormalized cellular adhesion in the epidermis of sc-SEs. The counterstaining for the cornified envelope-constituent loricrin shows with the advance of culture time ((**b**) 2, (**c**) 3, and (**d**) 6 weeks) a similarly narrow zone in the *stratum granulosum* demarcated as in skin (**a**). Bar: 50 μm.

Concomitant with normalized deposition of BM components, the polarization of basal cells improved as indicated by the confined localization of integrins around cells of the basal layer (Stark *et al.*, 2004). Their local distribution is a useful marker to assess the physiologic keratinocyte state during epidermal regeneration. All integrin subunits of normal human epidermis (α2, α3, α6, β1, and β4) were present in all stages of reconstructed epidermis in sc-SEs (starting with week one), revealing a regular decoration of the BM-zone (enhanced for α6 and β4) and particularly obvious by the apico-lateral deposition of β1 integrin on basal keratinocytes (Stark *et al.*, 2004). At later culture stages, the localization of the individual integrins got further normalized approaching features of resting epidermis *in situ* (Figure 7). Thus, in sc-SEs, the dermo-epidermal junction zone provided clear signs for stable tissue architecture with normalized differentiation.

ECM production and maturation in DEs

Corresponding to the regeneration and maturation of the epidermal compartment, the fibroblasts dispersed within the scaffold produced and deposited different ECM components, starting with those found in provisional wound matrix such as fibronectin, tenascin, and thrombospondin, which were detected by immunostaining from the first week on (see Figure 5). However, type I and III collagen, components of mature dermis, were also detected and increased in amount with culture time, as visualized by immunostaining and quantifed by Western blot (Stark *et al.*, 2004). The latter analyses clearly showed a positive impact on the presence of keratinocytes on the production of the different components. The density of most ECM components was obviously higher in the subepithelial zone and the deposits exhibited a fibrous texture, suggesting that local concentrations were comparable to normal human dermis. Thus, this model system exhibited cutaneous regeneration with intense interaction of epithelial and mesenchymal cells, underlining in return the important role of keratinocytes in the regulation of ECM synthesis.

Ultrastructural aspects of epidermal differentiation and BM

At the ultrastructural level, all typical features of epidermal differentiation were seen in sc-SEs already after 2 weeks (Stark *et al.*, 2004). These further normalized with time in culture to a regular epidermal phenotype. The cells in the granular layer harbored many mature stellate keratohyalin granules in contact with keratin filaments like in human skin

(Figure 8a). Transitional cells containing half-desmosomes were facing the corneocytes exhibiting an increasingly dense and homogeneous contrast with minor remnants of cell organelles, which is indicative of a normal orthokeratotic *stratum corneum.* Even more so, the maturation of a functional epidermal barrier, in addition to the localization of tight junction components, became evident by numerous lamellar bodies in the uppermost granular cells, some of them discharging their lipid content into the intercellular space (Figure 8a).

At the epidermal–matrix junction, the formation of a genuine BM with a *lamina densa* and hemidesmosomes linked to keratin filaments is already visible in some areas of 2 weeks sc-SEs, as well as some anchoring fibrils spanning the gaps between *lamina densa* and collagen bundles (Stark *et al*., 2004). However, these ultrastructural features of a complete BM became more regular and continuous along the total epidermal–dermal border zone with numerous anchoring fibrils in 6 weeks old cultures (Figure 8b). Those anchoring structures, mainly composed of type VII collagen, evolved earlier and more frequently in sc-SEs than in col-SEs (Stark *et al*., 2004), and steadily increased with culture time in accordance with the more intense and polarized immunostaining of type VII collagen (Figure 8c and see Figure 4b–d).

With regard to the 'dermal' texture underneath the junctional zone, sc-SEs exhibited a high density of banded collagen fibrils, mostly parallel to the BM (Figure 8b and c). This corroborated the findings on distribution and texture of the fibrillar collagens (type I and III) by immunofluorescence microscopy underlining proper neogenesis and assembly (see Stark *et al*., 2004).

DISCUSSION

Organotypic cocultures of keratinocytes and fibroblasts containing collagen hydrogels with incorporated fibroblasts have been applied as SEs in studies on various aspects of epithelial–mesenchymal interactions in epidermal regeneration and morphogenesis (Smola *et al*., 1993, 1998; Stark *et al*., 1999; Maas-Szabowski *et al*., 2000; Cerezo *et al*., 2003; Breitkreutz *et al*., 2004). Nevertheless, certain features of these conventional col-SEs remained unsatisfactory, such as limited lifespan, poor resistance against shrinkage as well as weakened anchorage of the epidermis on the collagen matrix. Moreover, for clinical application as grafted skin substitutes, it would be preferable to have larger and more

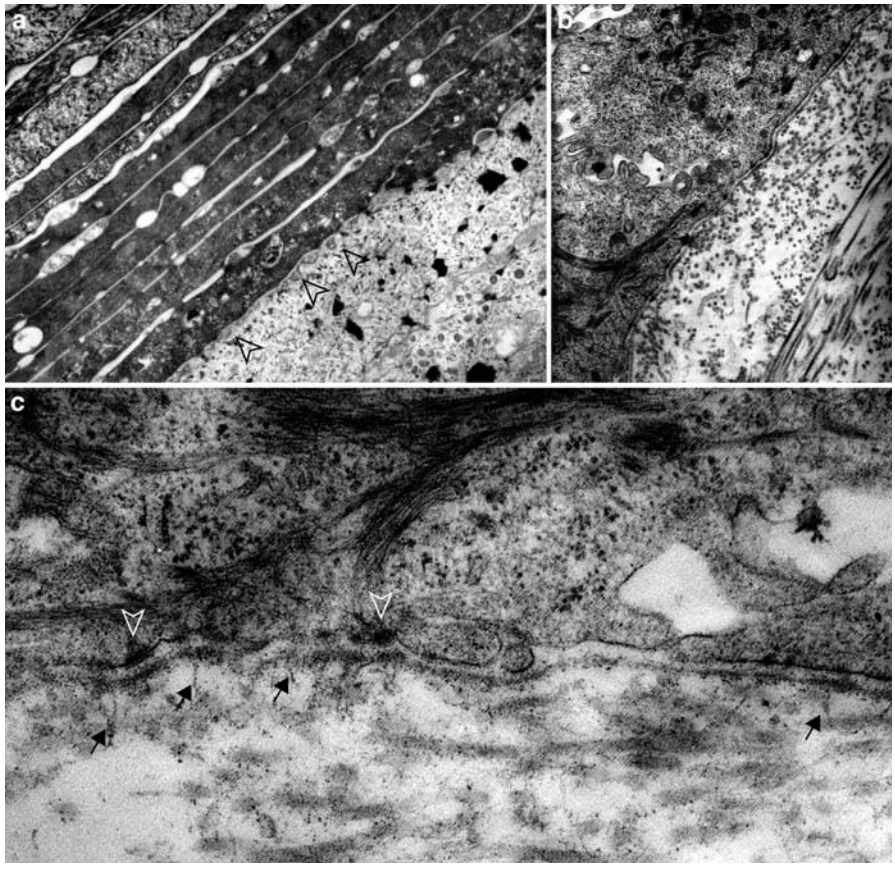

Figure 8. **Ultrastructural organization of the epidermis in 6-week-old sc-SEs.** (**a**) The thick *stratum corneum* exhibits increasingly dense contrast in the ''younger'' corneocytes facing a granule-rich *stratum granulosum* with numerous lamellar bodies along the upper membrane (arrow heads). (**b**) At the underside facing the DE the epidermis shows a continuous lining by a typical basememt membrane separating the epithelium from a dermal compartment rich in parallel-oriented collagen fibers. (**c**) At higher magnification, abundant anchoring fibrils (black arrows) connecting the *lamina densa* with collagen bundles as well as hemidesmosomes (white arrow heads) with inserting keratin filaments are obvious.

stable specimens and to avoid the xenogeneous collagen matrix due to the immanent possibility of immunological and infectious complications (Balasubramani *et al.*, 2001; Rennekampff and Hansbrough, 2001). With the aim to overcome these problems and to achieve normalization of epidermal histogenesis with features of tissue homeostasis, the objective was to produce and to functionally characterize improved SEs based on a novel type of a structurally reinforced DEs. These tissue-engineered DEs are produced within modified hyaluronic acid scaffolds colonized by dermal fibroblasts synthesizing authentic dermis-like ECM.

Similar concepts of SEs employing native, fibroblast produced ECM have been described before, using either nylon-meshes or collagen–chitosan–sponges as scaffolds (Yannas *et al.*, 1989; Fleischmajer *et al.*, 1991). In a different approach without scaffolds, long-term cultivated fibroblast sheets containing a considerable amount of self-assembled matrix have been utilized (Michel *et al.*, 1999). However, some obvious drawbacks are being encountered with these methods: (i) long precultivation times of 4–5 weeks are required for an organized ECM to form; (ii) the fibroblast sheets are delicate and difficult to handle; and (iii) because of its geometry and hardness, the nylon mesh scaffold is problematic to obtain useful, undamaged sections for morphological analysis. In particular, this latter point is a major obstacle for a comprehensive evaluation of epidermal histogenesis and ECM structure in SEs. All theses disadvantages are circumvented in the model presented here. The fabrication of DEs with Hyalograft-3D-scaffold requires not longer than 1 week precultivation before seeding of the keratinocytes. The implemented scaffold confers a considerable stability and resilience to the *in vitro* reconstructed skin, preventing shrinkage, facilitating handling, and last not least, allowing enlargement for industrial and clinical use. Finally, the mechanical properties of the fibrous Hyalograft-3D scaffold are in favor of sample processing to obtain full-size histological sections.

However, most important for this study, scaffold enforced DEs demonstrated excellent supportive functions for epidermal regeneration up to a state of tissue balance, herein by far surpassing col-DEs. At a first glance, SEs with both types of DEs exhibited a similar epidermal regeneration potential according to morphology, cell proliferation, and the full complement of differentiation markers such as K10, involucrin, and filaggrin. Nevertheless, a more in-depth analysis of individual keratin expression patterns provided clear evidence for advanced renormalization of the epidermis in sc-SEs, being significantly superior to col-SEs. One important example is the perfect co-expression of K1 and K10, both markers for early differentiation, that is restored on sc-DEs in contrast to persistent decoupled expression on col-DEs (Stark *et al.*, 1999). Being present in the epidermis only in minor amounts, K15 has largely been neglected in the past. Recently, however, it has gained interest as a potential marker for keratinocyte stem cells in the hair follicle (Lyle *et al.*, 1998). Under normal conditions, K15 is expressed in the basal keratinocytes of stratified squamous epithelia (Lloyd *et al.*, 1995; Waseem *et al.*, 1999), while there are controversial reports on overexpression or suppression in wound healing and respective models or in hyperproliferative diseases (Waseem *et al.*, 1999; Werner *et al.*, 2000). In col-SEs, K15 was present throughout several cell layers in the reconstructed epidermis at all time points analyzed, while a strict *in vivo*-like basal localization was seen in sc-SEs after 3 weeks pointing to re-establishment of physiologic, that is, homeostatic conditions.

The group of cytokeratins K6, K16, and K17 formerly considered as markers for hyper-proliferation is now associated with wound repair and keratinocyte activation and migration (Moll *et al.*, 1982; Stark *et al.*, 1987; Stoler *et al.*, 1988; McGowan and Coulombe, 1998). Supposedly, their functional role is to promote a reorganization of the keratin cytoskeleton to allow for keratinocyte migration during re-epithelialization (Paladini *et al.*, 1996). Whenever analyzed, K6, K16, and K17 have been detected in cultured SEs, including 3 weeks old col-SEs (Kopan and Fuchs, 1989; Stark *et al.*, 1999; Waseem *et al.*, 1999; el Ghalbzouri *et al.*, 2002). In the present study, we were able to show that after prolonged cultivation K16 was largely downregulated in sc-SEs. However, due to the considerable stability of keratin filaments in differentiating keratinocytes K16 usually persists for some time, especially in the *stratum corneum*. These findings strongly suggest a normalized regulatory state in the epidermis approaching tissue homeostasis.

Since the two types of SEs differ mainly in composition and organization of their DEs, it is conclusive, to attribute a special influence to the authentic ECM in sc-DEs. Support for this interpretation comes from transplantation studies showing that the *in vivo*-like reorganization of the dermal compartment is a prerequisite for complete normalization of epidermal differentiation (Breitkreutz *et al.*, 1997; Stark *et al.*, 1999; Tomakidi *et al.*, 2003). Therefore, we conclude that the authentic ECM present in sc-DEs provides adequate cues for reconstruction of a regularly polarized epidermis with normalized differentiation.

Another hallmark of normal epidermal histogenesis and stability is the formation of a structured BM. This highly specialized ECM-structure separates the epithelium from the underlying connective tissue, serves as a selective barrier, but at the same time provides firm epidermal anchorage to the dermis. Besides this important mechanical aspect, BM also confers instructive cues to the adhering germinative basal layer, which mediates and modulates factor-driven epithelial–mesenchymal interactions (Streuli *et al.*, 1995; Pujuguet *et al.*, 2000). The BM structure represents a supramolecular assembly of several macromolecules, including type IV collagen, laminins, perlecan, and nidogen (Timpl, 1996; Breitkreutz *et al.*, 2004). In different studies, production and deposition of BM components in cultured skin substitutes have been reported. However, a fully mature BM ultrastructure appeared only after 3 weeks of cultivation of col-SEs (Smola *et al.*, 1998; Stark *et al.*, 1999) or when DEs with ECM preformed in nylon meshes had been used (Contard *et al.*, 1993; Fleischmajer *et al.*, 1998). In our investigation, similar quantities and kinetics of deposition were observed for most BM components in col-SEs and sc-SEs, yet with the exception

that an earlier ultrastructural maturation of the BM in sc-SEs was correlated with strong deposition of type VII collagen. This constituent of the anchoring fibrils of the BM zone is supposed to be produced by the keratinocytes (Regauer *et al.*, 1990). In sc-SEs, this component is regularly deposited at the epidermal–dermal border zone as early as after 2 weeks in culture and the corresponding ultrastructural features clearly distinguished in 3 weeks old cultures, indicating that authentic dermal ECM favors complete BM maturation and stabilization.

Major players in the context of cell–matrix interactions are the integrins, first as sensors for the surrounding ECM environment and second as essential molecular devices for cell adhesion, migration, and ECM remodeling (Watt, 2002). Their regular distribution in epidermal keratinocytes is well established as are their altered patterns in wound healing and pathologic conditions (Tennenbaum *et al.*, 1996; Rikimaru *et al.*, 1997). Therefore, the irregular diffusion of non-cell-bound integrin reactivity into the subepithelial space, often observed in col-SEs for all the integrin subunits tested, points to a disturbed keratinocyte physiology. This is in line with the considerable membrane vesicle shedding from the basal cells that has been reported for these col-SEs (Stark *et al.*, 1999). Interestingly, the picture was strikingly similar in early stages of hetero-transplants on nude mice, when cells were grafted on the collagen hydrogels; integrin shedding vanished together with exogenous collagen upon replacement by host connective tissue (Breitkreutz *et al.*, 1997, 1998). Most deleterious in this context was that col-SEs were prone to splitting between tissue compartments loosening their structural integrity. This integrity is certainly required for the delicate equilibrium between keratinocytes and fibroblasts, that is, for tissue homeostasis. Accordingly, on sc-SEs we found a perfect and regular integrin pattern reflecting the *in vivo*-like epidermal architecture and correlating with composition and structural organization of the ECM in the tissue engineered dermis. Direct evidence is provided by immunostaining, showing a fibrous texture of native type I and III collagen deposited in the scaffold, which indicates a normalized fibrillogenesis compared to DEs with preformed collagen gels. Consequently, the normalization of ECM in sc-DEs is corroborated by the ultrastructural appearance of banded collagen fibrils whereas in collagen gels only structurally imperfect, less organized fibers are seen.

Quantitative evaluations of ECM components in the sc-DEs showed that dermal structural proteins such as type I collagen and constituents of the provisional matrix such as tenascin and thrombospondin are produced with different kinetics (Stark *et al.*, 2004). As generally established for regenerative processes (Singer and Clark, 1999), we found an outburst of transitory provisional matrix synthesis that is accompanied by a moderate but steady increase of type I collagen. Even more interesting was the observation that the presence of keratinocytes significantly intensified the amount of all ECM components tested, suggesting that the fibroblasts respond very sensibly to inductive stimuli from the epidermis. This is in agreement with studies reporting that keratinocytes are essential for the induction of elastic fibers in SEs (Duplan-Perrat *et al.*, 2000; Black *et al.*, 2005).

For technical reasons, we decided to utilize low concentrated fibrin gel in order to establish the fibroblast population in the scaffold. In addition to its initial function in mechanically stabilizing the fibroblasts in the DEs, a marked beneficial effect on the seeded keratinocytes and the subsequent epidermal morphogenesis became obvious. This observation is in line with several reports describing improved keratinocyte growth and performance and maintenance of ''stemness'' when combined with fibrin or plasma in clinical applications (Horch *et al.*, 1998; Pellegrini *et al.*, 1999; Ronfard *et al.*, 2000; Llames *et al.*, 2004). In our setting, however, the provisional fibrin matrix is replaced by newly synthesized authentic ECM, finally reassuming a physiological composition and structural organization that is as effective as native dermis in supporting epidermal histogenesis.

Further characterization will be necessary to define those key parameters of matrix biology in sc-DEs that are relevant for the positive impact on epidermal renormalization in these SEs. In this context, the functional role of the scaffold material and its influence on ECM production is still matter of speculation and has not yet been addressed by systematic experimentation. Probably, a major effect of the scaffold is to provide a sufficiently stable and rigid framework, thus enabling the DEs to build up an intrinsic tension that stimulates the fibroblasts to actively synthesize and organize their authentic ECM. This might exert a positive feedback on ECM synthesis. In fact, tensional forces have been found to induce a 'synthetic' phenotype in embedded fibroblasts with increased connective tissue synthesis while simultaneously inhibiting matrix degradation (Kessler *et al.*, 2001).

To summarize, the cultured SEs introduced here display an almost perfectly renormalized epidermal differentiation that exhibits many traits of tissue homeostasis. Their tissue engineered dermal part consists of a biomaterial scaffold, fibroblasts, and an authentic ECM of *in vivo*-like organization that sets the stage for the formation of a mature epidermal BM. In consequence, the correct assembly and spatial organization of the BM brings about a regular integrin pattern in the basal keratinocytes. These integrins, known to be engaged in outside-in-signaling, transmit external structural cues from the ECM into the keratinocytes, thus controlling epidermal differentiation, proliferation, and morphogenesis (Tennenbaum *et al.*, 1996; Breitkreutz *et al.*, 1997; Watt, 2002). Therefore, we consider our SE model being closer to the *in vivo*-situation than any other model described before. From our examinations, convincing evidences emerge for the instructive nature of those stimuli that emanate from a normal ECM and lead to an advanced normalization of epidermal regeneration. With the opportunity of prolonged cultivation times tolerated by these cultures a state of balanced proliferation and differentiation, that is, tissue homeostasis, was achieved. Homeostasis in the strict sense is maintenance of tissue mass and integrity by balanced cell gain and loss as it occurs *in vivo.* This is not achieved in these SEs *in vitro* – because there is no controlled loss of cells due to the absence of constant shedding of *stratum corneum* squames. However, the ongoing accumulation of keratinized squames has obviously no immediate effect on keratinocyte

proliferation and differentiation, because the normalization of major criteria is continuously improving during prolonged culture time. Nevertheless a potential impact of the accumulating layers of keratinized cells on keratinocyte growth and maturation as well as on epidermal barrier function cannot be excluded at present. Therefore, further studies including mechanical dislodgement of keratinized sheets have to demonstrate which effect shedding of the *stratum corneum* will have on the maintenance of epidermal homeostasis.

In conclusion, this model is ideally suited to elucidate the regulatory mechanisms of cell–cell and cell–matrix interactions controlling skin regeneration and homeostasis. Besides the impact of ECM on the control of epidermal differentiation, the role of the ECM microenvironment for patterning and maintenance of keratinocyte stem cells will be addressed exploiting this model (Bickenbach, 2003; Tumbar *et al*., 2004). A specific aspect of renormalized differentiation is the accomplishment of an *in vivo* like epidermal permeability barrier (Segre, 2003) what would represent an important progress in developing a relevant skin model for *in vitro* pharmaco-toxicological testing.

MATERIALS AND METHODS

Cell culture

Keratinocytes were isolated from adult human trunk skin, usually obtained from breast reduction surgery by a sequential treatment with thermolysin to separate the epidermis and with trypsin to release the cells according to Germain *et al*. (1993). Keratinocytes were plated at a density of 2–3 $\times 10^4$ cells per cm^2 on feeder cells (human dermal fibroblasts gamma-irradiated with 70 Gy, 1×10^4 cells per cm^2) in keratinocyte culture medium consisting of a 1:3-mixture of Ham's F12 and DMEM – traditionally named FAD medium (Wu *et al*., 1982) – supplemented with 5% fetal bovine serum (FBS), 1.8×10^{-4} M adenine, 10^{-10} M cholera toxin, 0.4 μg hydrocortisone, 5 μg insulin (all Sigma, Deisenhofen, Germany), and 1 μg human recombinant EGF (Promocell, Heidelberg, Germany) per ml. After one passage keratinocytes were cryo-preserved in aliquots of 2×10^6 cells. Growth potential was tested in feeder layer-based cloning assays, which also excluded fibroblast contamination. Five individual keratinocyte strains were used throughout this study.

Human dermal fibroblasts were isolated from explant cultures of de-epidermized dermis. The fibroblasts were expanded up to passage 3–4 in DMEM with 10% FBS and cryo-preserved. The experiments described here were performed with two fibroblast strains.

The use of human skin tissue from reduction surgery for the isolation of keratinocytes and fibroblasts was approved by the Ethics Committee of the Medical Faculty of the University of Heidelberg (notification no. 422005). According to the Declaration of Helsinki Principles, the patients had been informed and gave their written consent to the usage of their tissue for scientific research.

Scaffold-enforced dermal equivalents

The preparation of DEs based on type I collagen hydrogels (col-DEs) has been described previously in detail (Smola *et al*., 1998; Stark *et al*., 1999).

sc-DEs were fabricated using Hyalograft-3D, a fleece-like nonwoven fibrous material (about 1.2 mm thick) consisting of hyaluronic acid esterified with benzylic alcohol (Fidia Advanced Biopolymers, Abano Terme, Italy). Human dermal fibroblasts were introduced into the scaffold as fibrin gel suspension. The fibrin gel was prepared using Baxter-Tissucol/Tisseel, a two-component fibrin glue for surgical application (Baxter AG, Vienna, Austria). The fibrinogen component was diluted with PBS without Ca^{2+} and Mg^{2+} pH 7.0 to a fibrinogen concentration of 8 mg per ml, while in the second component the original thrombin concentration was reduced to 10 U using phosphate-buffered saline (PBS) as diluent. Circular pieces of Hyalograft-3D with 22 mm diameter were placed into 4.5 cm^2-Falcon-filter inserts (see above) and overlaid with 600 μl of a 1:1 mixture of diluted thrombin and FBS containing 9×10^5 fibroblasts. Immediately afterwards, 600 μl of fibrinogen dilution were added, thoroughly mixed, and evenly distributed over the whole area of the scaffold by gentle pipetting. After about 10 minutes at 37°C, a clot enclosing the fibroblasts had formed, filling the space of the scaffold and forming a smooth surface. Finally, these constructs contained 2×10^5 fibroblasts per cm^2, 4 mg fibrinogen and 2.5 U thrombin per ml. To prevent floating and to confine the central area for keratinocyte seeding, the constructs were covered by glass rings fitting into the membrane inserts and submersed in DMEM-medium with 10% FBS, 50 μg L-ascorbic acid and 1 ng rh transforming growth factor-β1 per ml. Furthermore, the medium (before keratinocyte seeding) was supplemented with 500 U aprotinin (Bayer, Leverkusen, Germany) per ml, which prevented precocious fibrinolysis by fibroblasts and keratinocytes. At the time of keratinocyte seeding, aprotinin was reduced to 200 U/ml and maintained at that level (medium changes every other day).

SEs on sc-DEs

The cocultures on sc-DEs were generated by seeding 1×10^6 keratinocytes inside the glass rings onto the equilibrated collagen gels yielding a density of 3×10^5 cells per cm^2. After submersed incubation overnight, the glass rings were removed and the medium level was lowered to the base of the DEs, thus exposing the culture surface to the air-medium interface. Cultivation was continued in FAD medium with 10% FBS, 10^{-10} M cholera toxin, 0.4 μg hydrocortisone, and 50 μg L-ascorbic acid, and 200 U of aprotinin per ml with medium change every other day.

Immunofluorescence and light microscopy

Specimens of SEs were embedded in Tissue Tek (Miles, Elkhart, IN), frozen in liquid nitrogen vapor. Cryostat-sections (5 μm) were mounted on 3-aminopropyl-triethoxysilane coated slides, air dried, and processed as described earlier (Stark *et al*., 1999). The sections were fixed in 80% methanol at 4°C followed by absolute acetone at –20°C and pre-blocked with 3% BSA in PBS. The incubation with the primary antibodies was carried out in a moist chamber at 37°C for 1 hour followed by 1 hour or overnight at room temperature. In general, double labelling was performed by applying two appropriate primary antibodies (from different species) simultaneously. For detection, species-specific, fluorochrome-conjugated secondary antibodies (Dianova, Hamburg, Germany; Molecular Probes, Eugene, OR) were applied mixed with bisbenzimide (Hoechst 33258, Sigma) for nuclear staining and specimens incubated for 30 minutes at 37°C followed by 30 minutes at room temperature. After washing thoroughly, the sections were mounted in Permafluor (Immunotech, Hamburg, Germany) and examined with a Leica

Table 1. List of primary antibodies

Antigen	Designation	Specification	Dilution	Source
Keratin 1	HK1 (H28.31)	gp polyclonal	1:200	D. Roop. Baylor College, Houston, TX
Keratin 10	—	rb polyclonal	1:3,000	D. Roop. Baylor College, Houston, TX
Keratin 10	DE-K10	mouse mAb	1:10	Progen, Heidelberg, Germany
Keratin 15	GP15.1	gp polyclonal	1:200	Progen, Heidelberg, Germany
Keratin 16	LL025	mouse mAb	1:10	I. Leigh, University Hospital, London, UK
Loricrin	—	rb polyclonal	1:1,500	D. Hohl, CHUV, Lausanne, Switzerland
Integrin β1	P4G11	mouse mAb	1:50	Chemicon, Temecula, CA
Occludin	—	rb polyclonal	1:25	Zymed, San Francisco, CA
Type IV collagen	—	rb polyclonal	1:400	Heyl GmbH, Berlin, Germany
Type VII collagen	—	rb polyclonal	1:5,000	L. Bruckner-Tudermann, Freiburg, Germany
EDA-Fibronectin	IST-9	mouse mAb	1:50	Philogen, Siena, Italy
Tenascin	—	rb polyclonal	1:800	Biomol, Hamburg, Germany
Vimentin	GP53	gp polyclonal	1:100	Progen, Heidelberg, Germany
5-BrdU	BU5.1	mouse mAb	1:10	Progen, Heidelberg, Germany

gp, guinea-pig; rb, rabbit.

DMRBE/RD photomicroscope equipped with epifluorescence illumination; micrographs were recorded with a CCD-camera (F-View 12) applying Analysis Pro 6.0-software (Soft Imaging Systerms, Münster, Germany).

The antibodies used in this study (listed in Table 1) were commercially available or generous gifts from colleagues as indicated.

Proliferation assay

Before harvesting SEs were incubated for 18 hours with a mixture of BrdU and 5-bromodeoxycytidine (BrdC; ICN Biomedicals, Irvine, CA; 65 μM each, added to the medium). Incorporation into the nuclei of replicating cells was detected by anti-BrdU-antibodies after methanol/acetone fixation and pretreatment with 1.5 M HCl (10 minutes at room temperature) followed by thorough rinsing in PBS; nuclei were counterstained as described before. Proliferative indices were determined by evaluating the fraction of BrdU-labeled nuclei in the basal cells in at least three vision fields of 2–3 sections of each culture specimen. Four separate experiments were undertaken with duplicate cultures for each time point and condition.

Histology and electron microscopy

For histology, SE-samples were fixed in 3.7% buffered formaldehyde pH 7.4 for 24 hours. To avoid distortion of the delicate specimens, they were embedded in 3% agar and postfixed for an additional 24 hours. After dehydration, the specimens were embedded in paraffin, and 4 μm-sections were stained with hematoxylin and eosin.

For ultrastructural analysis, small tissue blocs were fixed in 2.5% glutaraldehyde (0.05 M cacodylate buffer, pH 7.2) for 2 hours at room temperature and then in 2% OsO4 (0.1 M cacodylate buffer) for 2 hour. Subsequently, the specimens were incubated in 1% aqueous uranyl acetate for 18 hours, dehydrated and embedded in EPON 812. Ultrathin sections collected on copper grids were poststained with uranyl acetate and lead citrate, and examined on a Zeiss EM 10 transmission electron microscope adjusted to 100 kV.

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