# Human Eccrine Sweat Gland Cells Turn into Melanin-Uptaking Keratinocytes in Dermo-Epidermal Skin Substitutes

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Recently, Biedermann *et al.* (2010) have demonstrated that human eccrine sweat gland cells can develop a multilayered epidermis. The question still remains whether these cells can fulfill exclusive and very specific functional properties of epidermal keratinocytes, such as the incorporation of melanin, a feature absent in sweat gland cells. We added human melanocytes to eccrine sweat gland cells to let them develop into an epidermal analog *in vivo*. The interaction between melanocytes and sweat gland–derived keratinocytes was investigated. The following results were gained: (1) macroscopically, a pigmentation of the substitutes was seen 2–3 weeks after transplantation; (2) we confirmed the development of a multilayered, stratified epidermis with melanocytes distributed evenly throughout the basal layer; (3) melanocytic dendrites projected to suprabasal layers; and (4) melanin was observed to be integrated into former eccrine sweat gland cells. These skin substitutes were similar or equal to skin substitutes cultured from human epidermal keratinocytes. The only differences observed were a delay in pigmentation and less melanin uptake. These data suggest that eccrine sweat gland cells can form a functional epidermal melanin unit, thereby providing striking evidence that they can assume one of the most characteristic keratinocyte properties.

## INTRODUCTION

Recently, Biedermann *et al.* (2010) have demonstrated that human eccrine sweat gland cells are a source of epidermal stem cells. *In vitro* and *in vivo* studies showed the development of a multilayered interfollicular epidermis from cultured human eccrine sweat gland cells. This epidermis displayed structural and functional properties of normal human skin. The question, however, remains whether the former sweat gland cells are able to further adapt to their new environment and acquire functional key properties of epidermal keratinocytes, such as the interaction with melanocytes and the incorporation of melanosomes.

Melanocytes, derived from the neural crest, are important cells of the epidermis, located in its basal layer (Fitzpatrick and Breathnach, 1963; Jimbow *et al.*, 1993; Hornyak, 2006; Sommer, 2011). Together with keratinocytes, they form the epidermal melanin unit (EMU), a structural and functional unit that enables a complex system of melanin production, melanosome uptake by keratinocytes, and distribution to the supranuclear cytoplasm of the keratinocyte (Hearing, 1999; Boissy, 2003; Hearing, 2005; Plonka *et al.*, 2009; Ando *et al.*, 2012). The resulting melanin cap absorbs UV radiation, thereby ensuring epidermal protection against DNA damage (Thody 1999; Haass *et al.*, 2005; Costin and Hearing, 2007; Miyamura *et al.*, 2007; Yamaguchi and Hearing, 2009). The molecular and cellular mechanisms involved in melanosome uptake are not yet fully understood, but studies have identified the keratinocyte receptor protease-activated receptor-2 (PAR-2) to play an important role in melanosome phagocytosis by keratinocytes (Abdel-Malek *et al.*, 1992; Paine *et al.*, 2001; Scott *et al.*, 2001; Seiberg, 2001; Babiarz-Magee *et al.*, 2004; Abdel-Malek *et al.*, 2010; Choi *et al.*, 2011). By proteolytic cleavage at the N-terminal domain of PAR-2, a tethered ligand is exposed, which binds to the receptor and initiates intracellular signaling (Déry *et al.*, 1998).

On the basis of the evidence that sweat gland cells can convert into epidermal keratinocytes and can form an

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Abbreviations: DESS, dermo-epidermal skin substitutes; EMU, epidermal melanin unit; MITF, microphthalmia-associated transcription factor; PAR-2, protease-activated receptor-2; PBS, phosphate-buffered saline

epidermis, we hypothesize that they can also interact with melanocytes and integrate melanin, possibly utilizing the PAR-2 signaling cascade (schematically shown in Supplementary Figure S1 online).

To test this hypothesis, we added melanocytes to dermo-epidermal skin substitutes (DESS) containing either epidermal keratinocytes or human eccrine sweat gland cells. These melanocyte-containing DESS were then tested in an immuno-incompetent rat model, and structural components indicative of a functioning EMU were analyzed 3 weeks after transplantation.

## RESULTS

### *In vitro* studies

**Cultured human eccrine sweat gland cells express the PAR-2 receptor.** As described recently by Biedermann *et al.*, human eccrine sweat gland cells can develop into a multilayered epidermis. However, a fundamental property of epidermal keratinocytes was not shown in sweat gland–derived epithelial cells, i.e., their uptake of melanin. The transmembrane receptor PAR-2 was demonstrated to play an important role in melanin uptake by keratinocytes (Seiberg *et al.*, 2000). Furthermore, PAR-2 was expressed in a sweat gland cell line, NCL-SG3, as well as in intact eccrine sweat glands isolated from human skin samples (Bovell *et al.*, 2008, 2009). Here, the receptor is most likely to be involved in secretory functions (Bovell *et al.*, 2009).

To investigate whether sweat gland cells maintain PAR-2 expression in culture, thereby exhibiting one requirement for melanocyte interaction and melanin uptake, we isolated entire sweat glands from human skin biopsies. These consisted of a defined secretory and ductal domain (Figure 1a). When cultured, we obtained pure epithelial sweat gland cells (Figure 1b). In a next step, we applied reverse transcriptase–PCR analyses to cultured human eccrine sweat gland cells. As a control, the same method was applied to cultured epidermal keratinocytes. In both cell types, PAR-2 was detected, which proves the expression of the receptor on the messenger RNA level (Figure 1c). We confirmed these results on the protein level by revealing a specific band in both cell types by western blotting (Figure 1d).

**FACS analysis of epidermal cells after Hoechst incubation reveals pure keratinocyte and melanocyte fractions.** To examine the functional interaction of sweat gland–derived epithelial cells with melanocytes, we needed to combine both cell types in pure culture to analyze their behavior in transplantation assays. To achieve a homogenous culture of melanocytes, we applied an adapted Hoechst dye exclusion protocol from Goodell *et al.* (1996) to skin specimens. In the FACS analyses, after using the Hoechst protocol a cell population with a low intensity in Hoechst red and blue fluorescence, to our knowledge not yet described by others, was observed (Mel; Figure 2a), in addition to the other cell fractions (Bulk, Top, side population$_{Bulk}$, and side population$_{Top}$; Figure 2a). Visualizing the FACS data as forward-scatter (cell size) and side-scatter (granularity) plots (Figure 2b), the Mel fraction was relatively low in cell
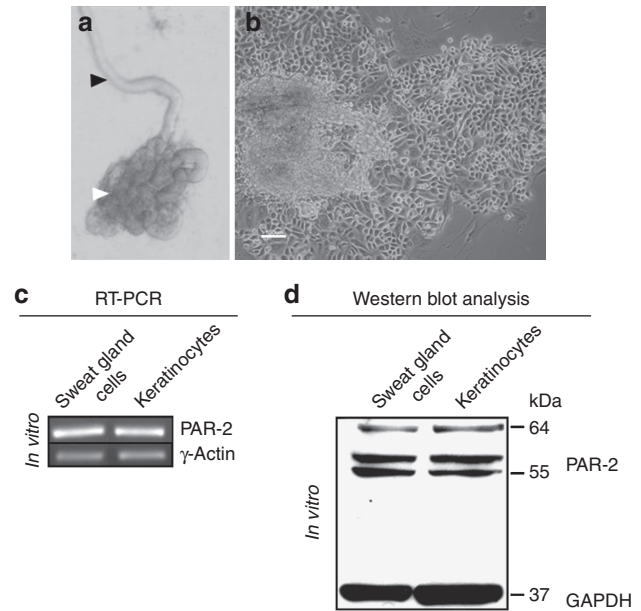


**Figure 1**. **Human eccrine sweat glands in culture and protease-activated receptor-2 (PAR-2) expression.** (**a**) A single, complete human eccrine sweat gland after enzymatic digestion. The sweat gland consists of a distinct ductal (black arrowhead) and secretory domain (white arrowhead). (**b**) Human eccrine sweat gland cells growing out of the glandular structure on cell culture plastic. (**c**) Reverse transcriptase–PCR (RT-PCR) revealing PAR-2 messenger RNA of cultured eccrine sweat gland cells and cultured primary keratinocytes. (**d**) Western blot analysis of PAR-2 protein isolated from cultured eccrine sweat gland cells and cultured primary epidermal keratinocytes. Bar (**b**) = 100 μm. GAPDH, glyceraldehyde-3-phosphate dehydrogenase.

number, representing 0.04% of the total epidermal cell preparation. It was clearly distinct from the other fractions as revealed by its higher granularity. Furthermore, in culture, the cells of the Mel fraction (Figure 2c) differed significantly in their morphology from the Bulk fraction (Figure 2d) and all other cell fractions. In contrast to the cobblestone-shaped keratinocytes, the cells of the Mel fraction displayed dendritic protrusions, suggesting these cells to be melanocytes, Langerhans, or Merkel cells. Immunofluorescence analyses using an anti-HMB-45 antibody that specifically detects melanosomes identified these cells as melanocytes (Figure 2e). In contrast, immunofluorescence analyses of cultured Bulk keratinocytes using the HMB-45 antibody did not stain these cells, whereas they were clearly recognized by the pan-cytokeratin aniserum (Figure 2f), highlighting the difference between the two cell types and revealing their origin.

To further confirm the purity of the Mel fraction identified by the Hoechst dye analysis, this cell population was again stained after culture for 6 days with an anti-HMB-45 antibody and subjected to FACS analysis. All cells stained positive for anti-HMB-45, identifying them as melanocytes (Figure 2g).

### *In vivo* studies

**Integration of melanocytes into eccrine sweat gland–derived DESS.** To compare the ability of eccrine sweat gland cells and interfollicular keratinocytes, to interact with melanocytes in a
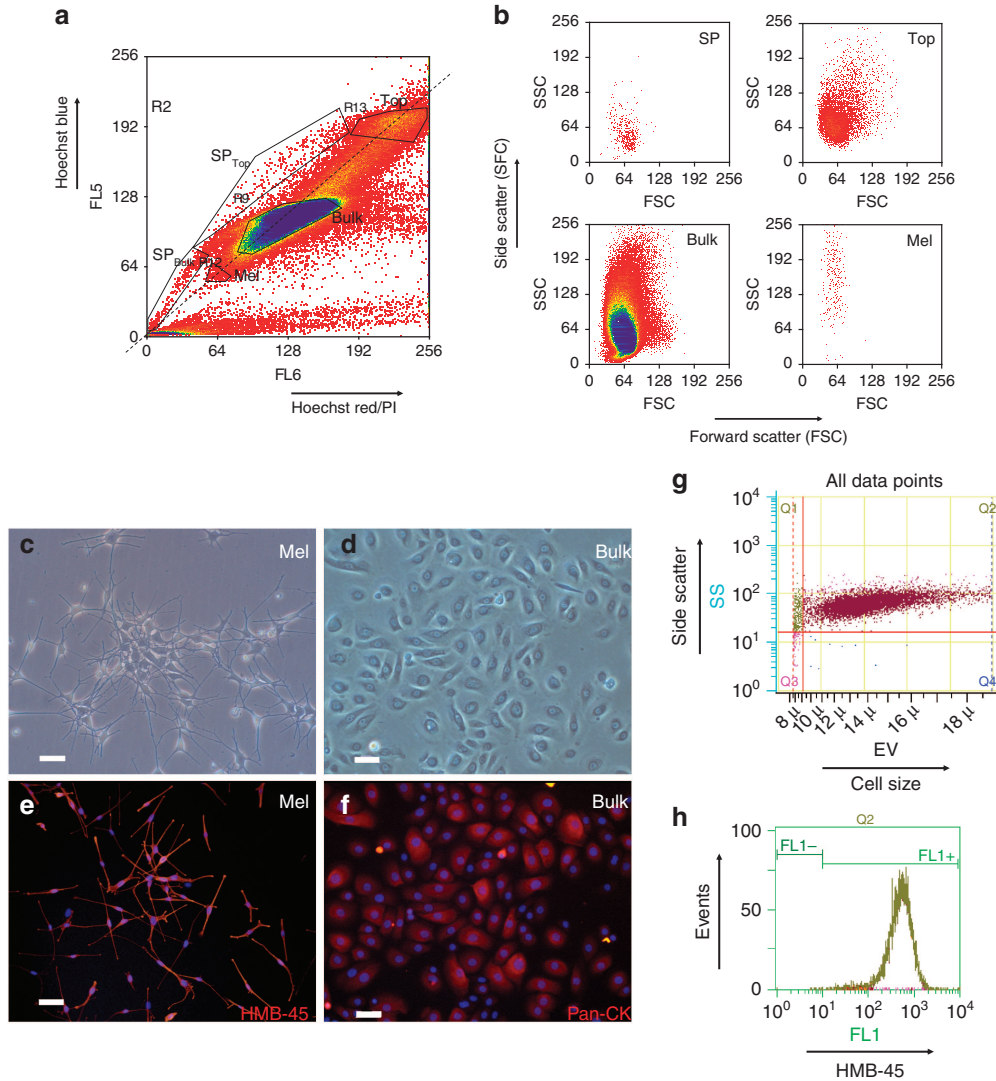
**Figure 2**. **Characterization of distinct epidermal cell fractions using a modified Hoechst dye exclusion protocol.** (**a**) FACS analysis after application of the Hoechst dye exclusion protocol reveals four different cell fractions: SP (side population, $SP_{Top\ and\ Bulk}$), Top, Bulk, Mel. (**b**) Visualization of the four fractions (SP, Top, Bulk, and Mel) according to cell size (forward scatter (FSC)) and granularity (side scatter (SFC)). (**c**) Culture of the Mel fraction in conventional cell culture. The cells have a stellate shape with dendritic protrusions. (**d**) The Bulk fraction grown on cell culture plastic. (**e**) The Mel fraction stained by an HMB-45 antibody (red), which recognizes melanosomes, revealing that these cells are melanocytes. Nuclei are stained by the Hoechst dye (blue). (**f**) A pan-cytokeratin antibody (red) shows the epithelial (keratinocyte) origin of the cells of the Bulk fraction. Nuclei are stained by the Hoechst dye (blue). (**g, h**) FACS analysis of the Mel fraction after staining with anti-HMB-45 antibody shows (**g**) a homogenous granularity and (**h**) specific binding of the antibody. PI, propidium iodide. All bars = 50 μm.

multilayered epidermis, we transplanted the corresponding DESS onto immuno-incompetent nude rats.

Macroscopically, we observed a homogenous darkening of the epidermis without hypo- or hyperpigmented spots in both types of transplants, 2–3 weeks after transplantation (Figure 3a and b). However, there was an obvious difference concerning the time point of appearance of pigmentation. Although the epidermal keratinocyte–derived constructs showed a macroscopically identifiable darkening already 1 week after transplantation (data not shown), the sweat gland cell–derived constructs started to darken only 2–3 weeks after transplantation.

After 3 weeks, the substitutes were excised and prepared for histological analyses. Hematoxylin/eosin staining con-

firmed the development of a stratified, multilayered epidermis in both the eccrine sweat gland cell and melanocyte-based DESS (Figure 3c) and the epidermal keratinocyte and melanocyte-based DESS (Figure 3d). No significant difference with regard to epidermal thickness, composition, stratification, or cornification could be seen in the two types of constructs.

To determine the integration and location of melanocytes added to both epidermal equivalents, further histological analyses were performed. We used the markers MITF (microphthalmia-associated transcription factor) and HMB-45 to visualize the melanocytes, and a staining for laminin-5 to delineate the basement membrane. In both constructs, melanocytes were located in the basal layer in
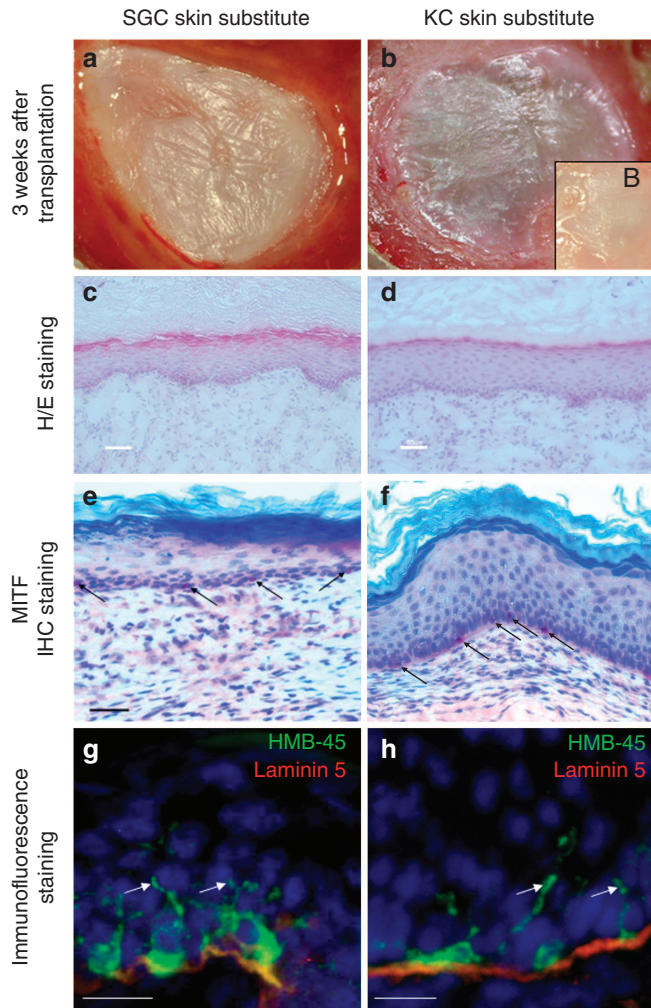
**Figure 3**. **Comparison of sweat gland cell (SGC)–derived and keratinocyte (KC)-derived skin substitutes containing melanocytes.** (**a**, **b**) Macroscopic appearance of (**a**) SGC and (**b**) KC skin substitutes 3 weeks after transplantation onto an immuno-incompetent rat. The insert (B) shows a dermo-epidermal skin substitute without added melanocytes for comparison. (**c**, **d**) Hematoxylin/eosin (H/E) staining of (**c**) SGC and (**d**) KC skin substitutes, respectively, 3 weeks after transplantation, both displaying a stratified and cornified epidermis. (**e**, **f**) Immunohistochemical (IHC) staining of (**e**) SGC and (**f**) KC skin substitutes revealing melanocytes (staining for microphthalmia-associated transcription factor (MITF), black arrows) evenly distributed throughout the basal layer. (**g**, **h**) Immunofluorescence staining of (**g**) SGC and (**h**) KC skin substitutes showing melanocytes (HMB-45, green) on the basement membrane (laminin-5, red) and melanocytic dendrites projecting to the suprabasal layers (white arrows). Bars (**c–f**) = 50 μm; (**g**, **h**) = 20 μm.

similar number and distribution (Figure 3e and f, black arrows), with melanocytic dendrites projecting to suprabasal layers of the epidermis (Figure 3g and h, white arrows).

***A functional EMU can be demonstrated in sweat gland cell–derived DESS.*** One melanocyte interacting with about 40 adjacent keratinocytes forms a functional EMU. To prove this melanocyte–keratinocyte interaction in our epidermal equivalents, further diagnostic tools were applied. Histological
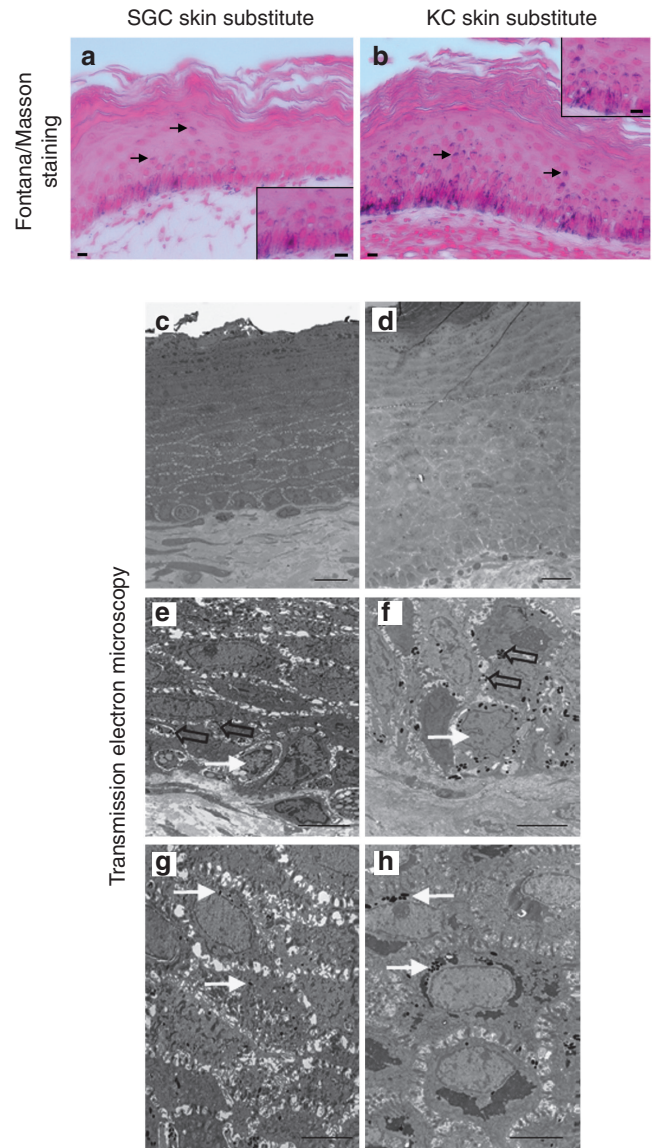


**Figure 4**. **Fontana/Masson staining and transmission electron microscopy of sweat gland cell (SGC) and keratinocyte (KC) skin substitutes.** (**a**, **b**) Fontana/ Masson staining of (**a**) SGC and (**b**) KC skin substitutes shows cytoplasmic melanin (black) in both melanocytes and KCs of both types of skin substitutes. A high concentration of melanin is detected in cells of the basal layer, whereas melanin in suprabasal cells is less abundant, but concentrated supranuclearly (black arrows). At a higher magnification, supranuclear melanin caps (black arrows) can be seen in SGC (inset of **a**) and KC (inset of **b**) skin substitutes. (**c**, **d**) A fully developed, stratified epidermis in (**c**) SGC and (**d**) KC skin substitutes. (**e**, **f**) Melanocytes (white arrows) are distributed throughout the stratum basale of (**e**) SGC and (**f**) KC skin substitutes. Dendrites containing melanosomes can be detected in both substitutes (hollow arrows). (**g**, **h**) Melanin granules (white arrows) are revealed supranuclearly in keratinocytes of both (**g**) SGC and (**h**) KC skin substitutes. Bars (**a**, **b**) = 10 μm; (**c**, **d**) = 50 μm; (**e–h**) = 5 μm.

sections were stained using the Fontana–Masson method, a procedure giving argentaffin granules and melanin a black color (Figure 4a and b). In our substitutes, we detected melanin in different strata throughout the epidermis, with a higher concentration of granules in the basal layer. In the suprabasal layers, melanin granules formed protecting

supranuclear caps (Figure 4a and b, arrows), as usually seen in normal human epidermal keratinocytes.

Electron microscopy was applied to specify melanocyte position and melanin deposition within the epidermal equivalents. Figure 4c and d shows a fully developed epidermis with all typical elements including a basement membrane and all epidermal strata in both constructs. At higher magnification, melanocytes were detected in the basal keratinocyte layer of both transplants (Figure 4e and f, white arrow). Furthermore, dendrites containing melanosomes were frequently detected (Figure 4e and f, black hollow arrows). Melanin was identified in epithelial cells of both origins (Figure 4g and h, white arrows), although not in equal amounts. A lower concentration of melanin granules was detected in keratinocytes derived from eccrine sweat gland cells. In both types of transplants, melanin was deposited as supranuclear caps (Figure 4g and h, white arrows). In summary, we found melanin not only in melanocytic dendrites but also in both types of keratinocyte, where it was deposited mostly in a supranuclear position.

## DISCUSSION

To our knowledge, this is a previously unreported study showing the ability of human eccrine sweat gland cells to interact with human epidermal melanocytes and to incorporate melanin into engineered DESS. In the global picture, human sweat gland cells and human epidermal keratinocytes behave similarly with respect to functional EMU formation. The sweat gland cells develop into a multilayered epidermis in which melanocytes find their place on the basement membrane. These melanocytes produce, store, and transport melanin, and the sweat gland cells phagocytose melanin and place it in a physiological supranuclear position. The following aspects deserve a detailed comment.

A pure melanocyte culture without contaminating epidermal keratinocytes was indispensable for our *in vivo* assays. To achieve this, we applied an adapted Hoechst protocol to an epidermal cell population, separating keratinocytes into different fractions (Goodell *et al.*, 1996; Terunuma *et al.*, 2003, 2007). We identified an additional small fraction consisting of melanocytes only. To our knowledge, this is a previously unreported description of the identification and extraction of pure melanocytes with this method, thus employing the Hoechst dye exclusion protocol for another so far unknown application.

As already shown by Biedermann *et al.* (2010), human eccrine sweat gland cells can form a stratified, multilayered epidermis, although there is a delay regarding the establishment of epidermal homeostasis. By adding melanocytes to our sweat gland-based constructs, we again found the above-quoted epidermis formation. In addition, we detected a close to normal incorporation of melanocytes into the DESS. Melanocytes positioned themselves physiologically along the basement membrane and developed dendritic protrusions into the upper layers of the epidermis. Melanocytes could not be seen in any other region than the basal layer. The end point of the physiological interaction between melanocytes and epidermal cells is characterized by incorporation and a topographically correct deposition of melanin in the epidermal cells. Histologic and ultra-structural analyses of our constructs 3 weeks after transplantation showed, in fact, melanin uptake and supranuclear deposition within keratinocytes derived from sweat gland cells. Apparently, and somehow surprisingly, sweat gland–derived keratinocytes (similar to interfollicular keratinocytes) have the potential to establish a photoprotection by interacting with melanocytes in a functional EMU. The crucial role of the supranuclear melanin cap in protecting DNA against UV radiation damage has long been recognized (Wood *et al.*, 1999; Boissy, 2003). Interestingly, a pigmented epidermal equivalent can also be cultured from hair follicle keratinocytes (Limat *et al.*, 1999; Liu *et al.*, 2011). Yet, the interaction of hair follicle keratinocytes and melanocytes is much less surprising given that there is a preexisting cooperation in their physiological function, i.e., the production of colored hair.

Taken together, it appears most remarkable that sweat gland cells stemming from skin appendages that naturally do not harbor melanocytes can transdifferentiate into keratinocytes and that these keratinocytes can commence a partnership with melanocytes to establish a functional EMU. A possible explanation for this phenomenon is that sweat glands (and hair follicles) arise from epidermal ridges during embryogenesis and therefore their cells may be able to restore previously silent epidermis-related functional programs (Hashimoto *et al.*, 1965; Holbrook and Minami, 1991; Hardy, 1992; Moll and Moll, 1992; Sato, 1993; Ersch and Stallmach, 1999). In analogy to the hair follicle bulge, it may be that certain sweat gland cells serve as a natural reservoir for keratinocytes, enabling the reconstruction of an epidermis following injury or some other stress situation. This is in line with reports showing that sweat gland–derived mesenchymal cells can influence and improve dermal regeneration during wound healing *in vivo* (Petschnik *et al.*, 2010; Danner *et al.*, 2012), emphasizing the ability of sweat gland cells (or sweat gland–associated cells) to exert functions distinct from their original ones.

Similarities between epidermal keratinocytes and sweat gland cells were also found on a molecular level. PAR-2 is known to play a crucial role in melanosome phagocytosis by keratinocytes (Scott *et al.*, 2001, 2003; Moretti *et al.*, 2009). When the expression of the receptor is blocked, no uptake of melanin into the cells can be detected (Choi *et al.*, 2011). PAR-2 was shown to be expressed in intact eccrine sweat glands isolated from human skin biopsies, as well as in a sweat gland cell line (Bovell *et al.*, 2008, 2009). Although the function of PAR-2 in sweat glands was shown to be associated with secretory events, its expression might become associated with melanin transfer and uptake when the cells assume a role as epidermal keratinocytes.

Furthermore, we do recognize differences in behavior of interfollicular epidermal keratinocytes and converted or reprogrammed eccrine sweat gland–derived keratinocytes, respectively. Macroscopically, sweat gland cell–derived constructs showed a less prominent pigmentation, and microscopically the quantity of melanin uptake and storage was clearly lower. It is unclear, whether the communication

between sweat gland–derived keratinocytes and melanocytes is less efficient because these cells are still about to convert to their new phenotype. This observation of sweat gland cell behavior is consistent with our previous findings describing a certain delay in the expression of epidermal markers in DESS (Biedermann *et al.*, 2010).

In summary, this experimental study shows the ability of human eccrine sweat gland cells—that have phenotypically converted into keratinocytes—to communicate and interact with melanocytes in almost the same way as original keratinocytes do. In particular, sweat gland cells fulfill several crucial requirements to form a classical EMU on a structural, functional, and molecular level. Although it is not their original function, human eccrine sweat gland epithelial cells can therefore be considered as an additional cell source to produce a near-natural epidermis in the context of skin tissue engineering.

## MATERIALS AND METHODS
### Isolation and culture of human keratinocytes, fibroblasts, eccrine sweat gland cells, and melanocytes
Human skin samples from the scalp, the retroauricular region, or foreskins were obtained after permission from the Ethics Commission of the Canton Zurich and after obtaining informed, written consent of parents or patients, in adherence with the Declaration of Helsinki Principles.

Human foreskins (from patients 1 to 16 years of age) were digested at $4\,^\circ C$ for 15–18 hours in $12\,U\,ml^{-1}$ dispase (BD Biosciences, Allschwil, Switzerland) in Hank's balanced salt solution containing $5\,\mu g\,ml^{-1}$ gentamycin (all from Invitrogen, Basel, Switzerland). The epidermis and dermis were subsequently separated. The epidermis was further digested in 1% trypsin and $5\,m M$ EDTA (all from Invitrogen) at $37\,^\circ C$ for a maximum of 3 minutes (Pontiggia *et al.*, 2009). Keratinocytes were harvested and cultured in serum-free keratinocyte medium containing $25\,\mu g\,ml^{-1}$ bovine pituitary extract, $0.2\,ng\,ml^{-1}$ epidermal growth factor, and $5\,\mu g\,ml^{-1}$ gentamycin (all from Invitrogen).

The dermal tissue was digested in $2\,mg\,ml^{-1}$ collagenase blend F (Sigma, Buchs, Switzerland) at $37\,^\circ C$ for $\sim60$ minutes. Dermal fibroblasts were grown in DMEM supplemented with 10% fetal calf serum (FCS), $4\,m M$ L-alanyl-L-glutamine, $1\,m M$ sodium pyruvate, and $5\,\mu g\,ml^{-1}$ gentamycin (all from Invitrogen).

Skin samples from the scalp or the retroauricular region were used to isolate sweat gland cells. These were digested in DMEM containing $12\,U\,ml^{-1}$ dispase and $2\,mg\,ml^{-1}$ collagenase blend F at $4\,^\circ C$ for 16 hours and at $37\,^\circ C$ for 4 hours, then transferred to a 10-cm dish containing DMEM, $15\,\mu g\,ml^{-1}$ gentamycin, and $0.3\,mg\,ml^{-1}$ penicillin/streptomycin (all from Invitrogen). The epidermis was removed with thin forceps and the dermis was chopped into small pieces ($<2\,mm^3$) with scissors. Sweat glands ''popped out'' because of shearing, were picked out under a stereomicroscope by aspiration with a micropipette, washed in a second dish with the same medium, and finally transferred to the culture dish (one or two glands per $cm^2$). To accelerate adhesion, only a minimal amount of Rheinwald and Green medium (three parts of DMEM and one part of Ham's F12, 10% FCS, $0.4\,\mu g\,ml^{-1}$ hydrocortisone, $5\,\mu g\,ml^{-1}$ insulin, $2\,n M$ triiodothyronine, $180\,\mu M$ adenine, $5\,\mu g\,ml^{-1}$ gentamycin, $1.4\,m M$ $CaCl_2$) was added (day 1) (all from Invitrogen). The sweat

glands were transferred to dishes containing feeder cells (Swiss albino 3T3 mouse fibroblasts, ATCC CCL-92) seeded at a density of 10,000 per $cm^2$ in DMEM, 10% calf serum, and treated with $10\,\mu g\,ml^{-1}$ mitomycin (all from Invitrogen) for 3 hours or cobalt irradiated at $60\,Gy$. Just before transferring the sweat glands, the medium was changed to Rheinwald and Green's medium. The day after isolation (day 2), one volume of medium was added, and at day 4 the first medium change was performed and $10\,ng\,ml^{-1}$ epidermal growth factor was added (Invitrogen). At day 4–5, the first outgrowing cells were visible and many colonies reached a diameter of $\sim5\,mm$ in 2–3 weeks. After this time, the feeder fibroblasts were removed by digestion with 0.1% trypsin, $1\,m M$ EDTA at $37\,^\circ C$ for 2–3 minutes. The sweat gland cells were washed twice in phosphate-buffered saline (PBS) and detached from the dish with 0.5% trypsin, $5\,m M$ EDTA at $37\,^\circ C$ for 5 minutes. The cell aggregates were reduced in size by repeated pipetting through a fine tip and transferred in equal volume of $3.75\,mg\,ml^{-1}$ soybean trypsin inhibitor (Invitrogen). After centrifugation, the cells were resuspended in Rheinwald and Green's medium with epidermal growth factor and counted.

Human melanocytes were isolated using Hoechst dye and a FACS analysis after trypsin digestion of the epidermis according to the keratinocyte isolation. The cells were cultured in melanocyte growth medium, containing 0.4% bovine pituitary extract, $1\,ng\,ml^{-1}$ basic fibroblast growth factor, $5\,\mu g\,ml^{-1}$ insulin, $0.5\,\mu g\,ml^{-1}$ hydrocortisone, $10\,ng\,ml^{-1}$ Phorbol Myristate Acetate (all from PromoCell, Heidelberg, Germany), and $5\,\mu g\,ml^{-1}$ gentamycin (Invitrogen).

### Hoechst 33342 staining
The trypsinized epidermal cell suspension was incubated with $5\,\mu g\,ml^{-1}$ Hoechst 33342 (Sigma) in serum-free keratinocyte medium and incubated at $37\,^\circ C$ for 2 hours. The concentration of the Hoechst dye was optimized by titration between 1 and $10\,\mu g\,ml^{-1}$. For a verapamil control, $150\,\mu M$ verapamil (Sigma) was also added. After 90 minutes, propidium iodide (Sigma) was added at $1\,\mu g\,ml^{-1}$ to the solution for a viability test and incubated for the remaining 30 minutes to complete the 2-hour incubation. After the incubation steps, cells were centrifuged at 1,100 r.p.m. at $4\,^\circ C$ and resuspended in $500\,\mu l$ serum-free keratinocyte medium. The cell suspension was strained a second time through a 50-μm cell strainer (Partec CellTrics, Münster, Germany) to avoid cell aggregates before the FACS analysis was performed.

### FACS analysis
FACS was performed immediately after Hoechst staining, using a DakoCytomation MoFLo flow cytometer equipped with an argon ion laser emitting at 488 nm and a UV laser emitting 351 nm (Dako, Baar, Switzerland). The forward-scatter and side-scatter signals were determined by the Ar ion laser, whereby the forward scatter was used as trigger. The emission of the Hoechst 33342 was detected using a 450/65 nm (''Hoechst blue'') and a 670/40 nm (''Hoechst red/PI'') narrow band-pass filter. The emitted signal was plotted in a Hoechst blue versus Hoechst red/PI dot plot to visualize the different fractions (Terunuma *et al.*, 2007). The data were collected in a linear mode. The data were acquired and analyzed with DakoCytomation Summit Software v3.3. Sorting experiments were performed at 60 p.s.i. sheath pressure at a drop rate of 5,000–10,000 cells per second, depending on the sample. The Bulk and Mel fractions were sorted and the cells subsequently cultured in their appropriate medium.

## Reverse transcriptase–PCR

Total RNA was prepared from cultured human keratinocytes or sweat gland cells, using an RNeasy mini kit (Qiagen, Hombrechtikon, Switzerland). The quality (purity and integrity) of extracted total RNA was confirmed using an Agilent 2100 Bioanalyzer with an RNA 6000 Nano Assay (Agilent Technologies, Basel, Switzerland). Fifty nanograms of total RNA from each sample were subjected to a one-step reverse transcriptase–PCR reaction using the RT-PCR Kit (Fermentas, St Leon-Rot, Germany) according to the manufacturer's instructions. Reverse transcription was carried out for 30 minutes at 50 °C and a hot start of 15 minutes at 95 °C was then included to activate HotStarTaq DNA polymerase in the reaction mix (Qiagen). The reaction contained 30 pmol of each primer of PAR-2 (PAR-2 s: 5′-TAGCAGCCTCTCTCTCCTGC-3′; PAR-2 as: 5′-TGAAGATGGTCTGCTTCAGC-3′), and 10 pmol of each primer of glyceraldehyde-3-phosphate dehydrogenase (GAPDH s: 5′-ACCACAGTCCATGCCATCAC-3′; GAPDH as: 5′-TCCACCACCCTGTTGCTGTA-3′; Microsynth, Balgach, Switzerland). PCR consisted of 30 cycles of denaturation for 50 seconds at 94 °C, annealing for 1 minute at 58 °C, and polymerization for 1 minute at 72 °C (Babiarz-Magee et al., 2004). The final extension step consisted of 10 minutes at 72 °C. PCR products were resolved by electrophoresis in 1% agarose gel and stained with ethidium bromide.

## Western blot analysis

Cells were washed twice with cold PBS and lysed at 4 °C in RIPA lysis buffer containing 20 mM Tris-HCl, pH 7.5, 150 mM NaCl, 5 mM EDTA, 1% (v/v) Triton X-100, 1 mM $Na_3VO_4$, protease inhibitor cocktail (Roche, Basel, Switzerland), and phosphatase inhibitor cocktail 1 (Sigma). Proteins were separated by SDS-PAGE under reducing conditions and transferred onto nitrocellulose membranes (Invitrogen). Membranes were probed with a mouse anti-PAR-2 mAb used in a dilution of 1:500 in 5% BSA (clone SAM11, Zymed Laboratories, San Francisco, CA) followed by horseradish peroxidase–conjugated rabbit anti-mouse secondary antibodies (Abcam, Cambridge, UK). Bound antibodies were detected by chemiluminescence (ECL, GE Healthcare, Buckinghamshire, UK).

## Organotypic cultures

The organotypic cultures were prepared as previously described (Pontiggia et al., 2009). In brief, hydrogels, consisting of rat collagen type I containing $1 \times 10^5$ human dermal fibroblasts (passage 1), were placed in cell culture inserts with membranes of 3.0 μm pore size (all from BD Falcon, Heidelberg, Germany). These dermal equivalents were grown in DMEM for 6 days to allow for gel contraction. On each dermal equivalent, $5 \times 10^5$ keratinocytes or sweat gland cells were seeded (passage 1). The melanocytes were seeded 1:5 with keratinocytes or sweat gland cells (Fukunaga-Kalabis et al., 2006). The DESS were cultured for 1 week in a 1:1 mix of DMEM and Ham's F12 containing the supplements described above and 2% FCS; during the second and third week, the FCS was reduced to 1%. After 4 days, the substitutes were raised to the air–liquid interface.

## Transplantation of cultured dermo-epidermal composites

The surgical protocol was approved by the local Committee for Experimental Animal Research (permission number 65/2009). DESS were transplanted onto full thickness skin defects on the backs of 10-week-old, female, athymic Nu/Nu rats (Harlan, Borchen, Germany). For anesthesia, isoflurane (Baxter, Volketswil, Switzerland) was used. The skin defects were encased by polypropylene rings, 27 mm in diameter (modified Fusenig chamber (Fusenig, 1994)), which were sutured with non-resorbable polyester sutures (Ethicon, Norderstedt, Germany). The transplanted grafts were covered with a silicon foil, excised after 21 days, and processed for sections or transmission electron microscopy analysis.

## Electron microscopy

For transmission electron microscopy analysis, tissue blocs ($\sim$1 mm$^3$) were prefixed in 0.1 M cacodylate buffer (Merck, Hohenbrunn, Germany), pH 7.3 containing 2.5% glutaraldehyde for 2 hours, washed in cacodylate buffer, postfixed with an aqueous solution of 1% $OsO_4$ and 1.5% $K_4Fe(CN)_6$ for 1 hour, dehydrated, and finally embedded in EPON 812 (Catalys AG, Wallisellen, Switzerland). Ultrathin sections (approximately 50–70 nm) were collected on copper grids, contrasted with 4% uranyl acetate and 3% lead citrate, and examined with a CM 100 transmission electron microscope (Philips, Eindhoven, The Netherlands). All reagents were from Sigma unless mentioned otherwise.

## Histological analysis

For cryosections, the DESS were embedded in OCT compound (Sakura Finetek, Staufen, Germany) and placed in dry ice, and then cut at −25 °C into 6- to 20-μm-thick sections. The transplanted constructs were stained with hematoxylin/eosin to assess the epidermal morphology. The Fontana–Masson technique was used to stain melanin (Weiner et al., 2007).

## Immunofluorescence staining

The immunostaining was performed by fixation and permeabilization of the sections in acetone at −20 °C for 5 minutes. The sections were air-dried and washed three times in PBS (Invitrogen), followed by blocking with PBS containing 2% BSA (Sigma) for 30 minutes. The blocked sections were incubated with the diluted antibodies at room temperature for 1 hour. Slides were then washed three times in PBS for 5 minutes and blocked for an additional 15 minutes. The staining of cell nuclei was carried out using 1 μg ml$^{-1}$ Hoechst 33342 (Sigma) after the last antibody incubation. Finally, the sections were mounted with Dako mounting solution (Dako, Baar, Switzerland) containing 25 mg ml$^{-1}$ of DABCO anti-quenching agent (Sigma).

The following antibodies were used for the immunofluorescence: HMB-45 (1:50, Dako), laminin-5 (clone P3H9-2, 1:100, Santa Cruz, Heidelberg, Germany), and pan-cytokeratin (clone AE1/AE3, 1:100, Dako). As secondary antibodies, FITC-conjugated polyclonal goat anti-mouse F(ab′)2 fragments (Dako) were used. For double immunofluorescence, the primary antibodies were pre-labeled with Alexa 555–conjugated polyclonal goat Fab fragments, according to the instructions of the manufacturer (Zenon Mouse IgG Labeling Kit, Molecular Probes, Invitrogen).

## Immunohistochemical staining

Immunohistochemistry was performed as described before (Eichhoff et al., 2010; Kiowski et al., 2012). Tissues were fixed in 4% paraformaldehyde and embedded in paraffin. Sections were

deparaffinized in xylene and rehydrated. For antigen retrieval, slides were heated with cell conditioner 1 (Ventana Medical Systems, Tucson, AZ). Staining was performed using the MITF antibody (clone 5 + D5, 1:50-Abcam, Cambridge, UK) and a detection kit supplied by Ventana (iVIEW DAB detection kit (Ventana)). Slides were counterstained with hematoxylin.

## Fluorescence microscopy

Fluorescence microscopy was performed using a Nikon Eclipse TE2000-U inverted microscope equipped with Hoechst, FITC, and TRITC filter sets (Nikon, Egg, Switzerland). For confocal imaging, the Eclipse TE2000-U was upgraded with a Nikon C1 Laser Scanning Microscope. A Helium–Neon Laser with 543-nm excitation was used for TRITC and an Argon Laser with 488-nm excitation was used for FITC. Images were processed with Photoshop 7.0 (Adobe Systems).

## CONFLICT OF INTEREST
The authors state no conflict of interest.

## SUPPLEMENTARY MATERIAL

Supplementary material is linked to the online version of the paper at http://www.nature.com/jid

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