De Novo Epidermal Regeneration Using Human Eccrine Sweat Gland Cells: Higher Competence of Secretory over Absorptive Cells

Luca Pontiggia[1], Thomas Biedermann[1], Sophie Böttcher-Haberzeth[1], Carol Oliveira[1], Erik Braziulis[1], Agnieszka S. Klar[1], Claudia Meuli-Simmen[1], Martin Meuli[1] and Ernst Reichmann[1]

In our previous work, we showed that human sweat gland-derived epithelial cells represent an alternative source of keratinocytes to grow a near normal autologous epidermis. The role of subtypes of sweat gland cells in epidermal regeneration and maintenance remained unclear. In this study, we compare the regenerative potential of both secretory and absorptive sweat gland cell subpopulations. We demonstrate the superiority of secretory over absorptive cells in forming a new epidermis on two levels: first, the proliferative and colony-forming efficiencies *in vitro* are significantly higher for secretory cells (SCs), and second, SCs show a higher frequency of successful epidermis formation as well as an increase in the thickness of the formed epidermis in the *in vitro* and *in vivo* functional analyses using a 3D dermo-epidermal skin model. However, the ability of forming functional skin substitutes is not limited to SCs, which supports the hypothesis that multiple subtypes of sweat gland epithelial cells hold regenerative properties, while the existence and exact localization of a keratinocyte stem cell population in the human eccrine sweat gland remain elusive.

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INTRODUCTION

Human epidermal homeostatic renewal and re-epithelialization after injury are promoted by keratinocyte stem cells, which are thought to reside in the basal layer of the epidermis (Strachan and Ghadially, 2008) and in skin appendages (Brouard and Barrandon, 2003; Blanpain and Fuchs, 2006). In the latter, eccrine sweat glands (ECGs) seem to act as major contributors to wound re-epithelialization (Rittie *et al.*, 2013). However, the only multipotent stem cell population of the skin characterized so far is located in the bulge region of the hair follicle (reviewed in Alonso and Fuchs, 2003; Cotsarelis, 2006). Sebocytes appear to be an early direct product of bulge cells (Frances and Niemann, 2012). Outer root sheath cells of hair follicles, originating from the bulge, differentiate *in vitro* into a fully stratified epidermis (Lenoir *et al.*, 1988; Limat *et al.*, 2003), and bulge stem cells contribute in mice to epidermal regeneration after wounding, yet not to homeostatic epidermal renewal (Ito *et al.*, 2005; Levy *et al.*, 2007). In contrast, sweat glands of mouse paw pads show a substantial turnover during both wound repair and homeostasis (Lu *et al.*, 2012).

In our previous work, we demonstrated that human ESG cells have the ability to reconstitute ''*de novo*'' a correctly stratified, interfollicular epidermis with a pronounced stratum corneum and a functional basal layer, which is firmly anchored to the basement membrane. The epidermal marker expression pattern was nearly identical to that of an epidermis derived from bona fide keratinocytes (Biedermann *et al.*, 2010). Additionally, sweat gland–derived epithelial cells also showed the ability to incorporate melanin, an important property of epidermal keratinocytes (Böttcher-Haberzeth *et al.*, 2012). Therefore, we concluded that sweat gland–derived epithelial cells represent a source of keratinocytes capable of growing a near normal autologous epidermis.

Anatomically, the ESG is a tubular structure, originating in the epidermis and blindly ending in the reticular dermis. It basically consists of two main segments: the distal absorptive and the proximal secretory duct. The absorptive duct is a stratified bi-layer composed of a peripheral and an inner luminal layer of absorptive cells (ACs). It can be subdivided into acrosyringium (intra-epidermal duct), straight (intradermal) duct, and intraglandular coiled duct (Figure 1a). The secretory duct consists of a pseudostratified single cell layer, which is composed of alternating clear, secretory, and dark, mucoid cells and is surrounded by myoepithelial cells (Langbein *et al.*, 2005). An interesting question raised by our previous work (Biedermann *et al.*, 2010) is: which subtype of sweat gland cells is involved in epidermal regeneration and

[1]*Tissue Biology Research Unit, Department of Surgery, University Children's Hospital, Zurich, Switzerland*

Correspondence: Luca Pontiggia, Tissue Biology Research Unit, Department of Surgery, University Children's Hospital, August Forel Strasse 7, Zurich CH-8008, Switzerland. E-mail: luca.pontiggia@kispi.uzh.ch

Abbreviations: AC, absorptive cell; DESS, dermo-epidermal skin substitute; ESG, eccrine sweat gland; SC, secretory cell

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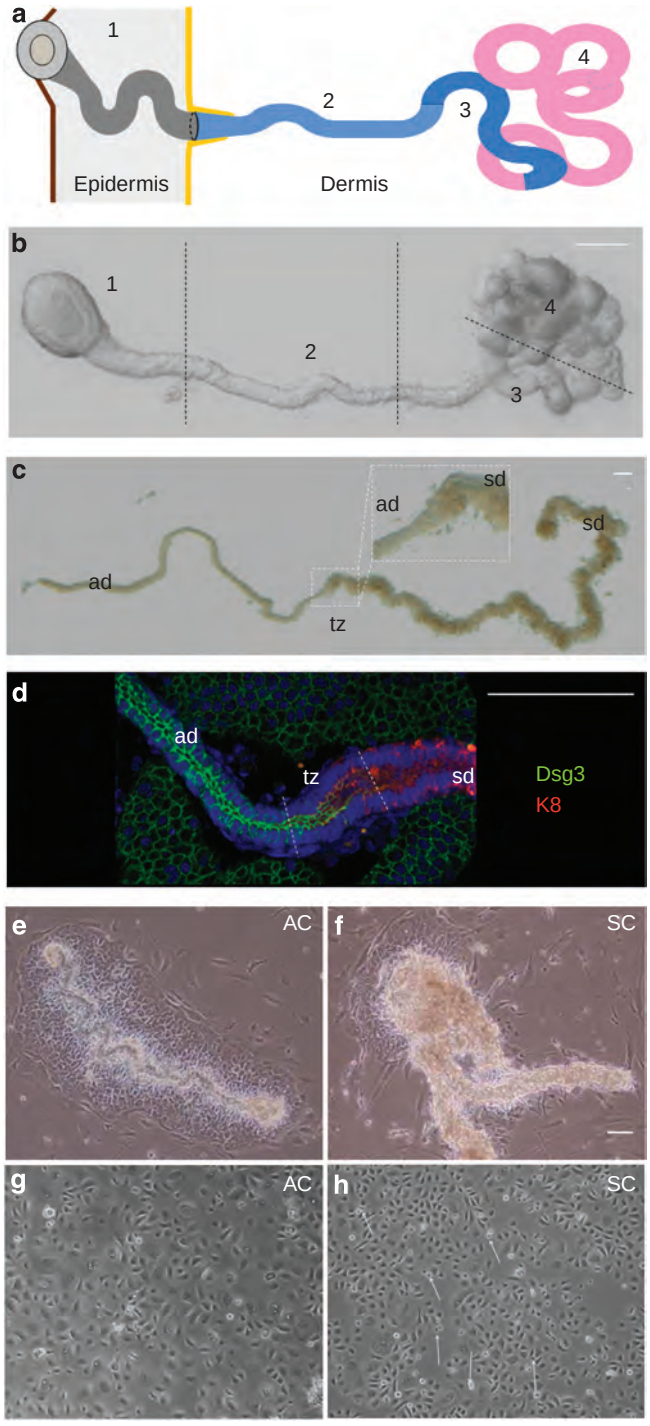

Figure 1. Isolation of pure populations of absorptive (ACs) and secretory cells (SCs) from the human eccrine sweat gland (ESG). (**a**) Illustration of the human ESG emphasizing four domains: Acrosyringium (intra-epidermal duct) (1), straight (2), and coiled (3) absorptive and secretory ducts (4). (**b**) Isolated ESG showing the same structural regions. (**c**) Streched gland showing absorptive (ad) and secretory ducts (sd) by light stereomicroscopy. The box illustrates a 2.6-fold magnification of the transition zone (tz) between the two main regions. (**d**) Whole-mount immunofluorescence staining of ESG. The luminal cells of the absortpive duct express desmoglein 3 (Dsg3; green), and the SCs express keratin 8 (K8; red). (**e**, **f**) Absorptive and secretory ducts cultivated on feeder cells for 6 days (note the outgrowing cells) and (**g**, **h**) after three passages in culture. The white arrows in **h** indicate some cell divisions. Bars = 100 μm.

maintenance? *In vivo* proliferative activity is found mainly in the absorptive domain of sweat glands *in vivo* (Langbein *et al.*, 2005; Li *et al.*, 2008; Lu *et al.*, 2012). However, the presence of a label-retaining (stem) cell (LRC) population is reported (in humans and mice) exclusively in the secretory domain (Nakamura and Tokura, 2009; Lu *et al.*, 2012).

The objective of our current study was to determine the role of human ESG cell subpopulations in skin regeneration and homeostasis and to test the hypothesis that the secretory domain of the human ESG may harbor an epidermal stem cell niche. To achieve this, we separately isolated cells from the absorptive duct (ACs) and from the secretory portion (secretory cells (SCs)) of the human ESG. We included ACs and SCs in organotypic cultures consisting of a stratified dermo-epidermal skin substitute (DESS). We show that cells gained from the secretory duct of the ESG are endowed with a clearly higher viability in culture and allowed to obtain high quality DESS *in vivo* and *in vitro* in a more reproducible way than ACs.

RESULTS

Isolation and discrimination of the absorptive and secretory domains of the ESG

We isolated human ESG (Figure 1b) from different body regions and from donors of different age groups (Supplementary Table S1 online). The transition zone between the dermal duct and secretory coil was clearly distinguishable using a stereo microscope (Figure 1c, tz). In addition to the visual discrimination, the two different gland domains were identified by whole-mount immunofluorescence using antibodies against desmoglein-3 (Dsg3), expressed exclusively in the absorptive duct (Figure 1d, ad), and keratin 8 (K8), limited to the secretory duct (Figure 1d, sd). To avoid possible contaminations and to obtain pure ACs and SCs, the short transition zone fragment was excised and eliminated. Absorptive and secretory fragments were cultivated separately on feeder cells until the outgrowing cells (Figure 1e and f and Supplementary Figure S7c online) had formed consistent colonies. The cells were passaged and expanded on collagen I–coated dishes in a serum-free, low calcium medium. The differences in size and morphology became evident after 2–3 passages: the AC population (Figure 1g, Supplementary Figure S2a, S2c, and S2e online) appeared inhomogeneous with rare mitotic and numerous big, terminally differentiated cells, whereas SCs (Figure 1h, Supplementary Figure S2b, S2d, and S2f online) were smaller in size, had a homogeneous cobblestone morphology, and revealed the presence of numerous small cell pairs as an immediate product of cell division (Figure 1h, white arrows).

Absorptive and secretory ducts express different markers, whose expression pattern is modified in culture

Many proteins are expressed specifically in SCs or ACs in normal skin (Supplementary Figure S1a online). We performed repeated analyses of the RNA and protein expression pattern by PCR and immunofluorescence to characterize the nature of the obtained cell cultures. We found that the original expression pattern of several markers was not maintained in culture.

For example, K17 and the epithelial sodium channel were expressed by both ACs and SCs in culture (Supplementary Figure S2a and S2b online), while their presence was limited to the absorptive duct in the normal human skin (Supplementary Figure S1a online). The same was observed for K5 (Supplementary Figure S2c and S2d online) and K77 (Supplementary Figure S2e and S2f online). The mRNA expression analyses of the muscarinic cholinergic receptor 3 and of the V0 subunit of the V-ATPase revealed minimal expression in both ACs and SCs in culture (Supplementary Figure S1b online), while these proteins were selectively expressed in the secretory duct *in vivo* (Supplementary Figure S1c online). Other markers were expressed in the original tissue but were downregulated in the cultured cell subpopulation (Supplementary Figures S1a and S6 online).

SCs showed higher proliferative and colony-forming efficiencies than ACs *in vitro*

Although it was not possible to identify markers to discriminate between SCs and ACs in culture, we observed specific properties of each of these two cell types in different assays. To compare the proliferative potential of the two ESG cell populations, SCs and ACs were plated in 24-well plates. The increasing number of viable cells was monitored by means of a colorimetric cell counting assay for 2 weeks. After an initial lag phase of 3 days, we observed a 2.8-fold higher proliferation activity in SCs during the following 7 days ($n=4$, Figure 2a).

We also compared the colony-forming efficiencies of SCs and ACs. The cells of five different donors were analyzed. Ten days after seeding, the number of colonies formed by SCs in each experiment was higher than the number of colonies formed by ACs. Figure 2b represents an example of a total of 15 analyses. No colony formation by ACs was seen in two experiments. Statistical analysis of AC and SC colony formation (number of colonies formed per 100 seeded cells) revealed a high variability (Figure 2c). The mean of all experiments (Figure 2d) showed a significantly higher colony-forming efficiency of SCs ($17\pm3\%$) compared with ACs ($3\pm3\%$; P-value <0.0001).

Epidermal thickness of *in vitro* DESS was larger when derived from SCs compared with ACs

As a next step, we studied the re-epithelialization potential of SCs and ACs.

Human eccrine SCs and ACs were seeded on collagen type I hydrogels previously populated with human fibroblasts. The growing skin substitutes were raised to the air/liquid phase for 3 weeks.

Hematoxylin/eosin staining showed that both SC and AC substitutes formed an epidermal multilayer of $\geqslant2$ layers or $\geqslant15$-μm thickness *in vitro* (Figure 3a and b′). However, an epithelium thickness of <35 μm was found more frequently among the AC substitutes (Figure 3b). The table in Figure 3c summarizes the results of these experiments and shows a better performance of SCs, which produced an epidermis with $\geqslant4$–10 keratinocyte layers and a thickness of >35 μm in four

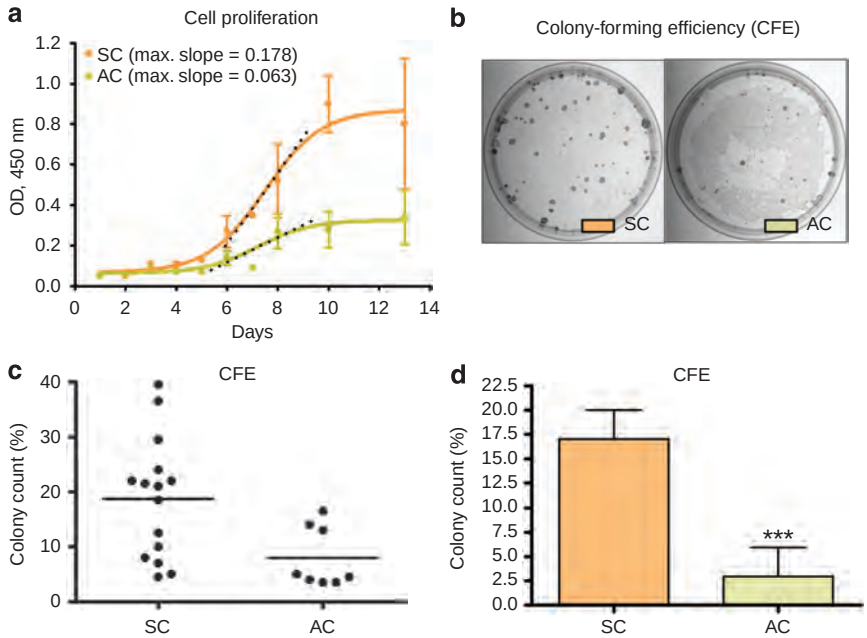

Figure 2. Proliferative and colony-forming efficiencies of absorptive (ACs) and secretory cells (SCs) *in vitro*. (**a**) The number of viable SCs or ACs was recorded in a colorimetric cell counting assay. Mean values and SDs at each time point and the maximal slope of the regression (dotted line) were calculated. The orange line marks the SC values and the green line the AC values. (**b**) Colony-forming assay, representative example: SCs or ACs were seeded and the formed colonies were stained by trypan-blue. (**c**) Number of colonies formed per 100 seeded cells (= colony-forming efficiency (CFE)). All 15 analyses (five triplicate experiments) are included in the plot. The horizontal lines denote the mean CFE values. (**d**) Mean of all experiments using normalized CFE values (see Supplementary Method section online). High statistical significance (***): P-value <0.0001. OD, optical density.

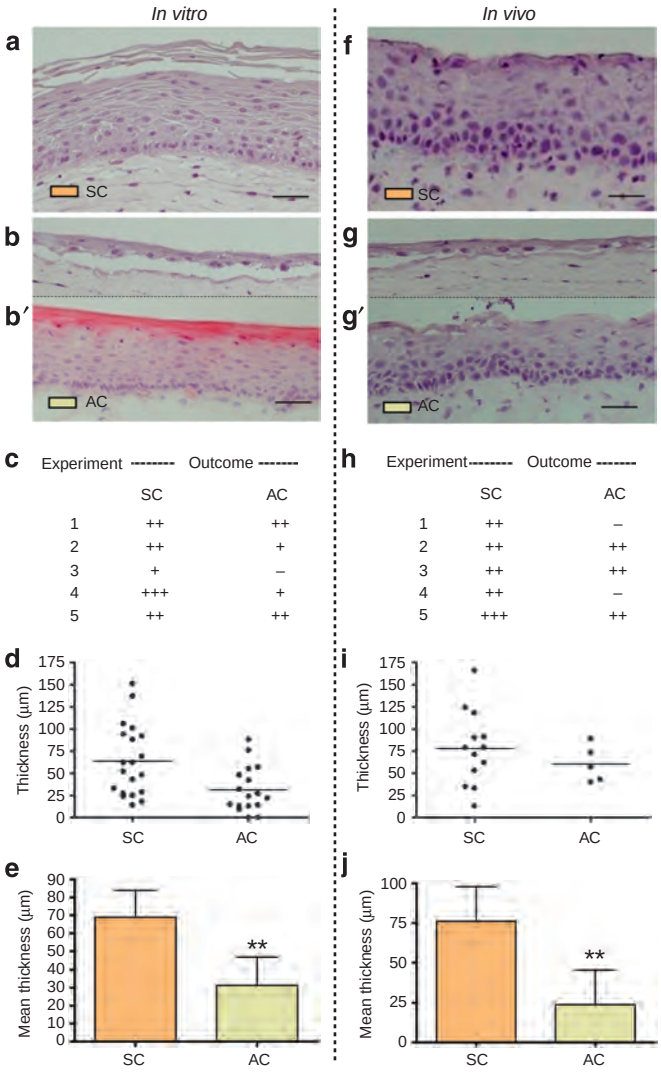

Figure 3. Generation of dermo-epidermal skin substitute (DESS) *in vitro* and transplantation onto immuno-incompetent rats. Analysis of hematoxylin/eosin staining of secretory (SC)- or absorptive cell (AC)-derived DESS 3 weeks after seeding of the epithelial cells (**a–e**, *in vitro*) and 3 weeks after transplantation onto immuno-incompetent rats (**f–j**, *in vivo*). (**a**) Example of stratification achieved *in vitro* with SCs. (**b**) Reduced stratification with ACs. (**b′**) Advanced stratification with ACs. (**c**) Area and length of the formed epidermis were measured. The mean thickness values of the five experimental series are summarized in the table: −, <15 µm (monolayer or nothing); +, 15–35 µm (1–3 cell layers); ++, 35–100 µm (4–10 cell layers); +++, >100 µm (>10 cell layers). (**d**) Assay variability of the epidermal thickness measured in all different single DESS. The horizontal lines denote the mean values. (**e**) Mean of all *in vitro* experiments using normalized values (see Supplementary Method section online). Statistical significance (**): *P*-value = 0.0045. (**f**) Example of advanced stratification achieved *in vivo* with SCs. (**g**) Poor and (**g′**) moderate stratification with ACs. (**h**) Area and length of the epidermis formed in the graft was measured. The table summarizes the mean thickness values of five experiments (scale as in **c**). (**i**) Assay variability of the grafted DESS as in **d**. (**j**) Mean of all *in vivo* experiments. Statistical significance (**): *P*-value = 0.0055.

experiments out of five. ACs reached such a performance in two out of five experiments only.

The epidermal thicknesses of all different single DESS were statistically analyzed (Figure 3d). We found a significantly larger mean thickness of substitutes derived from SCs when compared with those derived from ACs (mean thickness 69 µm vs. 31 µm, *P*-value = 0.0045; Figure 3e).

SCs demonstrate higher but not exclusive competence in DESS generation *in vivo*

DESS prepared with ACs and SCs in the epidermal compartment were transplanted onto nude rats to reproduce *in vivo* conditions. Three weeks after transplantation, the grafts were analyzed by hematoxylin/eosin staining (Figure 3f and g). SCs produced more than four epidermal layers with a mean thickness of >35 µm in all the experiments, whereas ACs achieved this performance in three experiments out of five only (Figure 3h). In the two failed experiments, ACs produced at least a monolayer before transplantation (macroscopic observation, not shown), yet the transplant was not able to grow and was lost for unknown reasons.

The variability of the obtained epidermal thickness of AC- and SC-derived substitutes was high (Figure 3i). However, the statistical analysis revealed a significantly higher mean epidermal thickness of 76 µm for SCs when compared with a mean thickness of 24 µm for ACs (*P*-value = 0.0055; Figure 3j).

Homeostasis and stratification of AC- and SC-derived DESS

To evaluate the grade of differentiation and homeostasis of the epidermal compartment of the *in vitro* and *in vivo* DESS, we analyzed the expression pattern of indicative proteins (Biedermann *et al.*, 2010). In the *in vitro* matured DESS from SCs and ACs, K1 was expressed from the stratum corneum down to the stratum spinosum (Figure 4a and g). After transplantation, this expression was extended to the first suprabasal layers, as it is seen in normal skin (Figure 4d and j). Involucrin expression was found in the stratum spinosum (Figure 4a, d, g, and j) of *in vitro* and *in vivo* SC- and AC-generated DESS, indicating an ongoing differentiation process.

The K15 expression of SC-derived *in vitro* substitutes (Figure 4b) was almost completely restricted to the basal layer, as in homeostatic conditions, whereas in AC substitutes it was also found in the stratum spinosum (Figure 4h). No expression of K15 in suprabasal layers was found in transplanted AC- and SC-derived substitutes (Figure 4e and k). Similarly, K19 expression was visible in the upper layers of the substitutes grown *in vitro* (Figure 4b and h). In the transplanted SC-derived substitutes, K19 was restricted to the basal layer (Figure 4e), and in three out of eight experiments K19 expression disappeared completely (not shown). In the transplanted AC-derived substitutes, K19 was not detectable in the basal layer (Figure 4k).

In vitro, the laminin 5 expression was strong but disorganized in SC-derived DESS (Figure 4c) and weak and scattered in AC substitutes (Figure 4i). The laminin 5 expression became more homogenous and similar to normal skin when DESS were transplanted (Figure 4f and l). The wound-healing marker K16 was detected in all the *in vitro* DESS (Figures 4c and i) but was downregulated in all *in vivo* experiments (Figure 4f and l).

Finally, all substitutes with >5 layers showed advanced stratification for both cell types, particularly when transplanted onto the rat.

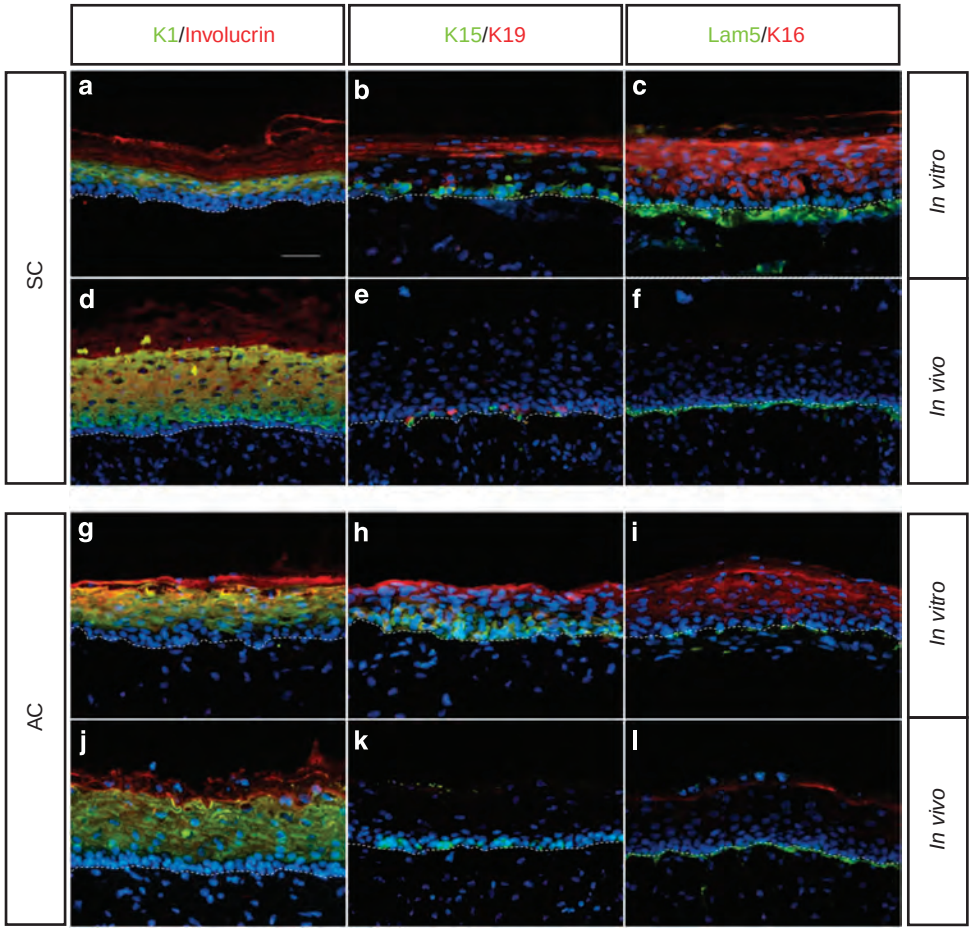

Figure 4. Epidermal stratification and homeostasis in sweat gland–derived dermo-epidermal skin substitute (DESS) *in vitro* and after transplantation onto immuno-incompetent rats. (**a**–**f**) Secretory cell (SC)-derived DESS, and (**g**–**i**) absorptive cell (AC)-derived DESS. (**a**–**c**, **g**–**i**) Typical epidermal differentiation of DESS *in vitro*, 3 weeks after seeding of the epithelial cells. (**d**–**f**, **j**–**l**) Epidermal differentiation of DESS *in vivo*, 3 weeks after transplantation. Immunofluorescence staining of cryosections using antibodies to: (**a**, **d**, **g**, **j**) K1 and involucrin, (**b**, **e**, **h**, **k**) K15 and K19, and (**c**, **f**, **i**, **l**) laminin 5 and K16. White dotted line: dermo-epidermal border. Bar = 50 μm.

DISCUSSION

The major role of human ESG is related to thermoregulation. However, the importance of this skin appendage is not limited to sweat production. Several observations lead to postulate the presence of a (multipotent) stem cell population in mammal glands: ESG cells show a certain capacity to form a multi-layered stratified epithelium in culture and *in vivo* (Lobitz *et al.*, 1954; Jones *et al.*, 1988; Miller *et al.*, 1998; Biedermann *et al.*, 2010; Böttcher-Haberzeth *et al.*, 2012), the proliferation marker Ki-67 is expressed in the absorptive duct (Langbein *et al.*, 2005), in mice LRCs are present in the secretory portion of the gland (Nakamura and Tokura, 2009; Lu *et al.*, 2012), and a major contribution of ESG cells to the re-epithelialization of epidermal wounds is reported in humans (Rittie *et al.*, 2013).

We aimed to test the hypothesis of the existence of a distinct stem cell population in a well-defined niche located in the secretory or absorptive duct of the ESG, which alone would be able to produce re-epithelialization. By comparing pure ACs with pure SCs, we demonstrate the superiority of SCs over ACs in forming a new epidermis *in vitro* as well as *in vivo*. We do this on two levels: First, the proliferative and colony-forming efficiencies *in vitro* are significantly higher for SCs. This appears to confirm the presence of a stem cell niche in the secretory portion of the gland. Second, as the stemness of epithelial cells is conclusively defined only by their ability to regenerate and maintain epidermis, we show by means of detailed functional analyses of DESS produced *in vitro* and *in vivo* that (1) the frequency of successful epidermal growth as well as the thickness of the produced epidermis is higher using SCs, while (2) both SCs and ACs are able to re-epithelialize properly, as confirmed by immunofluorescence staining.

In a previous work, we showed that the presence of K19-positive cells in the basal layer of normal or reconstituted epidermis is a sign of homeostatic conditions and may be related to the presence of self-renewing keratinocyte stem cells (Pontiggia *et al.*, 2009). Therefore, the presence of

K19-positive cells in most of the SC substitutes and in none of those generated with ACs may indicate the presence of a stem cell niche localized in the secretory domain. This would confirm our initial hypothesis and would be in line with the exclusive presence of LRCs in the secretory portion of mouse and human sweat gland (Nakamura and Tokura, 2009; Lu *et al.*, 2012).

However, as ACs are also able, although to a lesser extent, to produce a qualitative epidermis under certain circumstances, the existence of a particular niche of stem cells may be questioned. An alternative hypothesis may be proposed: SCs and ACs are only apparently fully differentiated cell populations and maintain different (reduced) regenerative capacities instead. In fact, as shown in our previous work, keratinocytes are able to quickly regenerate a new epidermis within 3 weeks, while sweat gland–derived cells seem to require a longer ''reprogramming'' time. They produce good quality epidermis, but they do not express all epidermal markers (such as K2e) after 7 weeks (Biedermann *et al.*, 2010). Human ESG develop at around gestational week 15–20 by budding from the epidermal basal layer and protruding into the dermis (Ersch and Stallmach, 1999). At this stage, most of the migrating cells may have stem cell character (Lu *et al.*, 2012). These stem cell properties may be progressively, but never completely, repressed by feedback mechanisms (Lander, 2009) during the process of differentiation, which is different for ACs and SCs.

Even though the observed differences between ACs and SCs can be explained through different reprogramming abilities, the simultaneous presence of a stem cell population that behaves according to the traditional models (Kaur, 2006; Simons and Clevers, 2011) cannot be excluded *a priori.*

The aspect of the purity of the SC and AC populations also deserves a comment. To avoid cross-contaminations, we excised and eliminated the intermediate fragment between secretory and absorptive duct, the transition zone. It could be argued that by doing so we may remove exactly the site in which the stem cell niche is localized. Yet, preliminary experiments with cells taken from the transition zone produced intermediate results to those made with ACs and SCs (Supplementary Figure S3 online). For this reason, we believe this cell population to be a mixture of ACs and SCs and not to contain a particular stem cell population, as cautiously postulated by Langbein *et al.* (2005). Consequently, we did not further analyze the transition zone cell population.

A related issue concerns the heterogeneity of the cell populations gained from the secretory domain and the absorptive duct of the ESG. The secretory domain is a pseudostratified epithelium composed of clear and dark cells (with different functions) and surrounded by myoepithelial cells (Saga, 2002). Similarly, the absorptive duct is composed of an inner or luminal and an outer or basal cell layer (Saga, 2002), but the composition of the cell population obtained from this fragment is undetermined. Therefore, different cell types with different characteristics (Collie *et al.*, 1985; Reddy *et al.*, 1992) may be present in our DESS, and it is not entirely clear which cell types contribute to epidermal regeneration. All these different types of cells may be endowed with different regenerative potentials. Their characterization would help to elucidate the mechanisms regulating ''stemness'' and differentiation of ESG cells and potentially lead to the identification of a new stem cell niche in the ESG.

Our attempt to identify clear and dark cells in SC cultures by immunofluorescence staining with markers reported in the literature (Zancanaro *et al.*, 1999; Clunes *et al.*, 2004; Kabashima *et al.*, 2008) showed that clear cells are probably one but not the single component of the SC population (Supplementary Figure S6 online). Because of the instability of the markers under *in vitro* culture conditions, it was not possible to draw a more precise conclusion on the identity of SC subpopulations.

Lu *et al.* (2012) confirm the presence of a LRC population in the secretory domain of the ESG and strongly suggest the presence of different unipotent progenitors supporting each lineage within the ESG, including myoepithelial cells. More recently, Leung *et al.* (2013) were able to isolate LRC with myoepithelial characteristics.

The presence of myoepithelial cells in our SC-derived DESS cannot be excluded. We observed their presence in the SC cultures during the outgrowing phase (Supplementary Figure S7b–e online). After the expansion phase with different medium conditions, the analysis of marker expression pattern typical for myoepithelial cells indicates that the SC population used for DESS formation may still include myoepithelial cells (Supplementary Figure S7 f–l online). As myoepithelial cells posses the potential for epithelial differentiation (Schon *et al.*, 1999), they may influence the stratification process and contribute to the differences between SC- and AC-stratifiying abilities.

From a clinical point of view, the most important challenge remains the *de novo* generation of ESG in DESS. Unfortunately, after full-thickness skin loss, appendages are also irreversibly damaged and can therefore not regenerate. Understanding the mechanisms that regulate the fate of epithelial (stem) cells may allow to mimic the biological processes leading to the formation of the gland during embryonic development. In this respect, Sheng *et al.* (2009) show in humans that regeneration of functional ESG is possible by reprogramming bone marrow–derived mesenchymal stem cells (Sheng *et al.*, 2009). In an ''ontologically inverse'' process, these cells are able to form a secretory domain, which protrudes absorptive ducts up to the epidermis. In mice, cells purified from both the absorptive duct and from the secretory domain retain the potential of *de novo* generation of new glandular structures (Lu *et al.*, 2012).

In conclusion, this is, to our knowledge, the first study comparing the regenerative potential of pure ACs and SCs and showing the superiority of SCs over ACs in forming a new multilayered functional epidermis *in vitro* as well as *in vivo*. In our opinion, our data do not sustain the hypothesis of a specific stem cell niche located in the secretory domain of the ESG but rather a different reprogramming ability of the two main cell populations. Further work is needed to characterize the different cell populations in the human ESG, their developmental and differentiation status, and function.

MATERIALS AND METHODS

Primary cell cultures

The study was performed in adherence to the guidelines of the WMA Declaration of Helsinki. Ethics approval was obtained from the Ethics Commission of the Canton Zurich, and written informed consent was given by patients or parents.

Human skin samples from different body regions (Supplementary Table S1 online) were obtained from patients aged 2–64 years. Keratinocytes and fibroblasts were isolated and cultured as previously described (Pontiggia *et al.*, 2009). Sweat gland cells were isolated and cultured according to Biedermann *et al.* (2010) with following modifications: after dispase/collagenase-I digestion of the skin samples, sweat glands were micro-dissected under a stereomicroscope (SMZ 1500, Nikon AG, Egg, Switzerland). The transition zone between the two different gland portions was visually identified and rejected. Resorptive and secretory fragments were cultivated as previously described for whole glands (Biedermann *et al.*, 2010).

Proliferation assay

Cells in passage p1–p2 after isolation were seeded in a collagen-I-coated 24-well-plate. For each cell type, quadruplicates of 20,000 cells per well were prepared. Every 2 days, the cell number was determined by means of a colorimetric cell counting assay (Cell-Counting-Kit, Sigma, Buchs, Switzerland). The experiment was repeated four times with cells from different donors. Mean values and SDs at each time point were calculated, and the maximal slope was calculated after non-linear regression curve fitting.

Colony-forming assay

Two hundred ESG cells, passages 1–3, were seeded on mitomycin-treated 3T3 mouse feeder fibroblasts in six–well culture dishes. The experiment was run in triplicate for each cell type for five different donors. After 10 days, single colonies were visible. The feeder layer was removed by short incubation in 0.1% trypsin, 1 mM EDTA (Invitrogen, Basel, Switzerland). The dishes were washed in phosphate-buffered saline (PBS), and 0.2% trypan-blue solution (Sigma) was added for 10 min. After profuse washing, pictures of the dishes were taken by standard light microscopy (Eclipse TE2000-U, Nikon), and the colonies were manually counted and statistically analyzed. The number of colonies formed per 100 seeded cells was reported in Figure 2c and d.

Organotypic cultures and transplantation of cultured dermo-epidermal composites

Compressed hydrogel production, organotypic cultures, and transplantation experiments were performed as previously published (Biedermann *et al.*, 2010; Braziulis *et al.*, 2011). We compared, in different experimental series, 15 different cell populations isolated from different body regions and from donors of different ages (Supplementary Table S1 online).

Antibodies

Antibodies to the following markers were applied for immunofluorescence stainings: From Dako (Baar, Switzerland): K19 (clone RCK108); from Chemicon (Millipore AG, Zug, Switzerland): K16 (clone LL025) and K1 (clone LHK1); from Santa Cruz (Labforce AG, Nunningen, Switzerland): laminin-5 (clone P3H9-2) and K15 (clone spm190), from LabVision (P.H.Stehelin&CIE AG, Basel, Switzerland): involucrin (clone SY5); from Genetex (Biozol, Eching, Germany): K8 (clone B391), and from Zymed (Invitrogen): Dsg3 (clone 5G11). FITC-conjugated polyclonal goat F(ab')$_2$ fragments directed to mouse immunoglobulins were from Dako. For double immunofluorescence, some of the primary antibodies were pre-labeled with Alexa 555-conjugated polyclonal goat F(ab')$_2$ fragments, according to the instructions of the manufacturer (Zenon Mouse-IgG Labeling Kit, Invitrogen).

Histology and immunofluorescence microscopy

Histology, three-color immunofluorescence stainings, and confocal microscopy of DESS were performed as previously described (Biedermann *et al.*, 2010).

Whole-mount stainings were performed as follows: after dispase/collagenase digestion of the skin, the epidermis was gently separated from the dermis. Sebaceous gland ducts of different lengths remained anchored in the epidermis. After fixation in 4% paraformaldehyde for 2 hours and permeabilization in 2% BSA and 0.5% Triton X-100 in PBS for 30 minutes, the first antibody incubation (Dsg3) was performed over night at 4 °C in the same buffer, followed by three washing steps of 1 hour in 0.2% Tween in PBS. In the same way, the FITC-conjugated secondary antibody and the Alexa-555 pre-conjugated third antibody (K8) were incubated for 2 hours and followed by three washing steps of 20 minutes each. During the first of the three washing steps, 1 μg ml^{-1} Hoechst 33342 (Sigma) was added for 5 minutes. Finally, the epidermis was transferred to a slide covered with mounting solution and a coverslip for confocal analysis. The immunostaining procedure for cells in culture was as previously described (Pontiggia *et al.*, 2009).

Statistical analysis

For the colony-forming assay, five triplicate experiments were performed. The mean number of colonies formed per 100 seeded cells was calculated. For the DESS formation, hematoxylin/eosin–stained cryosections were microscopically analyzed. By means of a computer software (ImageJ Software, Wayne Rasband, National Institutes of Health), the area of the formed epidermis and the length of the DESS were measured. The quotient of the two values describes the mean thickness of the DESS in each single probe. Means, SDs, and significances (unpaired *t*-test) of all normalized experiments were calculated using the GraphPad Prism (GraphPad Software, La Jolla, CA). Significance was set at an α-level of 0.05. For normalization of the data, see Supplementary Methods online. The tables with the original and normalized data are shown in Supplementary Figures S4c, S5a, and S5b online.

CONFLICT OF INTEREST

The authors state no conflict of interest.

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SUPPLEMENTARY MATERIAL

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

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