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Eccrine sweat glands associate with the human hair follicle within a defined compartment of dermal white adipose tissue*

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Summary

Background Eccrine sweat glands (ESGs) are critical for thermoregulation and are involved in wound healing. ESGs have traditionally been considered as separate skin appendages without connection to the pilosebaceous unit (PSU). However, recent preliminary evidence has encouraged the hypothesis that the PSU and ESG are more interconnected than previously thought.

Objectives To re-evaluate the morphology of human skin adnexa with an integrated three-dimensional (3D) perspective in order to explore the possible interconnections that the PSU and the ESG may form.

Methods A systematic 3D reconstruction method of skin sections, direct visualization of human scalp follicular unit transplant grafts and a scalp strip *ex vivo* were used to validate and further explore the hypothesis.

Results We demonstrate that the coiled portion of most ESGs is morphologically integrated into the PSU of human scalp skin and forms a structural unit that is embedded into a specific, hair follicle-associated region of dermal white adipose tissue (dWAT). This newly recognized unit is easily accessible and experimentally tractable by organ culture of follicular units and can be visualized intravitally.

Conclusions We propose a model of functional human skin anatomy in which ESGs are closely associated with the PSU and the dWAT to form a common homeostatic tissue environment, which may best be encapsulated in the term 'adnexal skin unit'. The challenge now is to dissect how each component of this superstructure of human skin functionally cooperates with and influences the other under physiological conditions, during regeneration and repair and in selected skin diseases.

What's already known about this topic?

- Eccrine sweat glands (ESGs) are traditionally regarded as being separate from the pilosebaceous unit (PSU).
- However, it has recently been hypothesized that human scalp ESGs may actually form an integral part of the PSU.
- The dermal white adipose tissue (dWAT), formally called the upper subcutis, is known to differ functionally from subcutaneous adipocytes and to be associated with hair follicles.

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Conflicts of interest
None to declare.

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What does this study add?

- We show by three-dimensional reconstruction imaging and intravital dye staining that the coiled portion of scalp ESGs is morphologically closely associated with neighbouring hair follicles and that both form a structural adnexal skin unit that is largely embedded into the dWAT.
- The location of the ESG coil can be identified macroscopically in a distinct area, close to the connective tissue sheath of the hair follicles, right below the sebaceous gland.

What is the translational message?

- The described observation is clinically relevant as it mandates to evaluate and consider individual skin appendage structures and their embedding into the dWAT as one morphological unit of human skin.
- In this unit, each element may influence the other under physiological conditions, during wound healing and in selected skin diseases.
- Pharmacological manipulation of one component of the unit may therapeutically impact on another.

Eccrine sweat glands (ESGs) are skin adnexal structures distributed over most of the human body, which provide an effective mechanism of thermoregulation.[1–3] In fact, ESGs are by far the most effective mechanism to combat the risk of death due to body temperatures exceeding 43 °C, which may provoke protein denaturation. Representing a relatively recent acquisition in the evolution of the skin in some mammals, mainly primates,[4] widespread distribution of ESGs throughout the integument offers these species the ability to tolerate and maintain physical activity even under conditions of extreme heat.[5]

The epithelium of ESGs also harbours stem cells,[6,7] while the ESG stroma is the richest source of pluripotent nestin-positive progenitor cells in human skin.[8,9] ESGs are also involved in wound healing, and functional human epidermis can be generated *ex vivo* from eccrine epithelium.[10,11]

As opposed to apocrine sweat glands, which open into the hair follicle (HF) epithelium and excrete their contents to the infundibulum, ESGs open to the epidermal surface through the acrosyringium, located in the interfollicular epidermis. Indeed, ESG ducts maintain this interfollicular position also at the level of the papillary dermis.[10] This is why ESGs have traditionally been set apart in their morphology and function from the pilosebaceous unit (PSU), which conventional wisdom holds to be composed of the HF, the arrector pili muscle (APM), the sebaceous gland and, where present, the apocrine sweat gland.[12]

However, when following the whole trajectory of the ESG from the skin surface to the hypodermis, we recently observed that the secretory coiled portion of most ESGs progressively moves closer to the HF and becomes spatially closely associated with it.[13] In the current study we have therefore systematically followed up the hypothesis that these ESGs actually form an integral component of the PSU,[13,14] using a three-dimensional (3D) reconstruction methodology on human scalp skin sections, and observation of follicular unit (FU) transplant grafts and intravital dye staining of whole-mount human-scalp skin strips *ex vivo*.

Materials and methods

Six normal human-scalp skin specimens were obtained from two routine autopsy cases (one male and one female) with normal clinical hair distribution and density. The patients had no clinical signs of androgenic alopecia. Specimens 3 × 1·5 cm in size were obtained from the temporal area from both sides of each patient. Samples were formalin fixed, paraffin embedded and stored at the Biobanc-Mur, integrated into the Spanish Biobanks Network, PT13/0010/0018 (www.redbiobancos.es) and registered on the Registro Nacional de Biobancos (#B.0000859). In addition, 30 FU extractions and one vertical scalp strip were obtained from the occipital scalp area of six male patients (five FUs from each patient and a scalp strip from one patient) during routine hair transplant. These patients gave written consent for donating material for research.

Histology

Autopsy material was properly oriented to obtain serial transverse sections from the surface down to the deep adipose tissue. For histology, 5-μm-thick sections were cut from formalin-fixed, paraffin-embedded tissue blocks. Alternate serial tissue sections were used for haematoxylin and eosin (HE) staining (even-numbered sections) or immunohistochemistry (odd-numbered sections). One of the autopsy blocks was used for vertically oriented sections. Serial vertical sections of FUs were stained with HE.

Immunohistochemical staining

Immunohistochemical staining was performed on deparaffinized tissue sections with a cocktail of keratin antibodies that clearly stain both the ducts and the acini of the ESGs and all epithelial structures of the HF, with the exception of the inner root sheath (irrelevant to our study), using an automatic platform (Dako Omnis) with prior heat-induced antigen retrieval in high pH buffer and the Envision Flex Kit (Agilent-Dako, Glostrup, Denmark). Primary antibodies used were broad-spectrum monoclonal mouse antihuman cytokeratins (clone AE1/AE3, Agilent-Dako) and the monoclonal mouse antihuman muscle actin antibody (clone HHF35, Agilent-Dako).

Three-dimensional reconstruction methods

For manual reconstruction, HE-stained transverse sections were scanned with an Aperio Scanscope (Leica Microsystems, Milton Keynes, U.K.). A group of FUs was selected from each whole-slide image and photographed. These microphotographs were serially assembled in layers using Adobe Photoshop (CS2 version; Adobe Systems Software Ireland Ltd, Dublin, Ireland), and the eccrine ducts and acini were coloured. Visual follow-up of the serial sections allowed us to represent the spatial disposition of HFs and adnexal structures.

For computer-assisted 3D reconstruction, immunohistochemical AE1/AE3-stained sections were scanned with the Aperio Scanscope. Using the Aperio Image Scope software, images were selected and photographed with the same methodology used for HE-stained sections. An automatic 3D reconstruction was performed using SynapseWeb Reconstruct version 1·1·0·0 software (http://synapseweb.clm.utexas.edu).

Stereomicroscopic analysis

FUs were analysed under a stereomicroscope (Motic, Kowloon Bay, Hong Kong). As ESGs are not visible in native human skin *ex vivo*, we examined them in frozen, vertical HE sections after being demarcated with India ink as a histological reference marker. This was performed at the HF region suspected to contain the ESG coil, thus in a region below the sebaceous gland that presented a foamy appearance resembling 'microbubbles'. In addition, the vertical scalp strip obtained from hair transplant surgery was stained with the supravital dye methylene blue, which can highlight human ESGs *in vivo* and *ex vivo*.[14]

Results

Most eccrine sweat gland secretory coils in human scalp skin are morphologically integrated into the pilosebaceous unit

On vertically oriented sections only a few ESGs appear to have their secretory portion located close to the outer root sheath of the anagen terminal HFs (Fig. 1a), or in the immediate proximity of the inferior pole of the club hair of telogen HFs (Fig. 1b), embedded into the dermal white adipose tissue (dWAT). This explains the conventional view that ESGs and PSUs are separate and unassociated anatomical structures in human skin. However, on some vertical sections lacking HFs, ESGs were also observed to be localized just below the APM, as highlighted by actin immunostaining (Fig. 1d).

Next, serial transverse sections stained with HE were prepared to demonstrate clearly the interrelation that the FUs maintain with the ESGs and the dWAT, and to follow the complete trajectory of the ESGs around anagen terminal scalp HFs. Sections separated in layers using Adobe Photoshop software were manually aligned, thus making it possible to reconstruct an image in which the intraepidermal acrosyringium displayed a clear interfollicular pattern, and the ESG ducts remained almost parallel to HFs at the level of the papillary dermis, as previously described.[10] However, at deeper skin levels the ducts progressively approached a defined HF in their immediate vicinity, with the coiled ducts and the ESG acini contacting the connective tissue sheath of that HF (Fig. 1c). This area of maximum proximity of the ESG and HF was typically located below the sebaceous gland and the insertion of the APM.

Direct observation of methylene blue-stained scalp strips under the stereomicroscope clearly revealed that ESGs are routinely located below the sebaceous glands, in the direct vicinity of HFs, with both structures being embedded into the cone-shaped dWAT that invaginates the reticular dermis (Fig. 2a). Thus, the secretory coil of the ESG is morphologically integrated with the PSU of human scalp skin and forms a structural unit located in a cone-shaped 3D compartment at the dermohypodermal interface, embedded into the dWAT (Fig. 2b).

Hair follicle-associated eccrine sweat glands remain attached to explanted follicular unit punch grafts

Next, we argued that, if ESGs are indeed as closely associated with human scalp HFs as suggested above, it should be possible to find them in single, individually extracted FU hair transplant grafts. In fact, in 80% of the 30 FUs removed from occipital scalp skin, using a 1-mm biopsy punch (from six hair transplant patients), we identified a distinct area below the sebaceous glands that showed a characteristic foamy appearance under the stereomicroscope that resembled 'microbubbles' (Fig. 3). When stably demarcated with India ink, this foamy-appearing FU area was shown by histology to correspond to the ESG coil, richly embedded into adipose tissue (Fig. S1; see Supporting Information). Measurement of the distance between the epidermis and the superior portion of the eccrine coil, estimated using direct stereomicroscopic observation of 23 FUs, gave values of 2–3 mm (Fig. 3).

Moreover, in order to identify further the depth and position of the eccrine coils, three FUs were horizontally cut immediately below the easily identified sebaceous gland, and both resulting fragments were analysed separately. The inferior fragment routinely demonstrated the presence of an eccrine

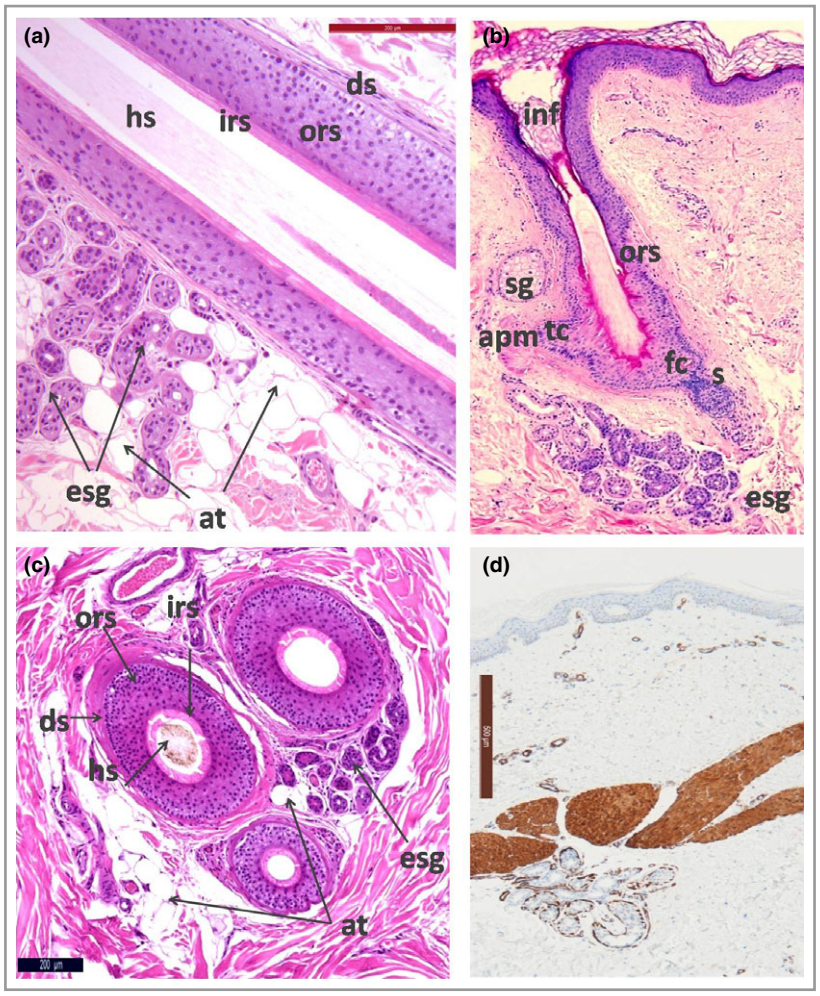

Fig 1. (a) Vertical haematoxylin and eosin stained sections of skin show the coiled portion of the eccrine sweat gland (esg) close to the hair follicle (HF) and embedded in dermal white adipose tissue (at). ors, outer root sheath; irs, inner root sheath; hs, hair shaft; ds, dermal sheath. (b) Vertical section of a telogen HF showing an infundibulum (inf) and a secondary germ (s). Eccrine sweat glands rest below the outer root sheath (ors) of the follicular club (fc), the arrector pili muscle (apm), the trochanter (tc) and the sebaceous gland (sg). (c) Transverse, horizontal section of a follicular unit with three anagen HFs that show the acinar portion of the eccrine sweat gland close to the HFs and immersed in adipose tissue. (d) On this actin-immunostained vertically oriented section of skin, the acinar portion of the eccrine sweat glands demarcated by actin-positive myoepithelial cells appears below the arrector pili muscle. This section, in which no HFs are present, although they should appear on consecutive sections, illustrates one of the reasons why eccrine sweat glands are not traditionally associated with the follicular unit.

coil embedded into the dWAT (Fig. 4). Therefore, the 'microbubbles', which indicate the presence of ESGs, with an identical location described in skin histological sections, provide an important and previously overlooked, but easily detectable morphological clue that indicates the presence of an ESG in an FU. Most importantly, this clue documents how intimately this part of the ESG is associated with the HF, in the region just below the sebaceous gland.

Three-dimensional computer-based reconstruction confirms the presence of hair follicle-associated eccrine sweat glands

For a more precise observation of the entire ESG trajectory without any subjective interpretation we used a computer-based 3D reconstruction method. In order to avoid any possible confusion with other dermal structures that may morphologically simulate ducts or acini, such as blood vessels, we used transverse sections immunostained with a pankeratin antibody (AE1/AE3). In these sections the epidermis and all the epithelia that were present at the subepidermal level were visible, namely the HF, the sebaceous gland and the ESG. The images created by this staining method followed a biphasic stained/nonstained pattern, ideal for computer manipulation.

Digital images obtained in this manner revealed that eccrine ducts were directed to a defined skin area below the sebaceous glands, where they form the secretory coil of the ESG (Fig. 5). The acinar portion of the ESGs appeared to be integrated within the FUs with prolongations into the spaces between HFs of the same FU.

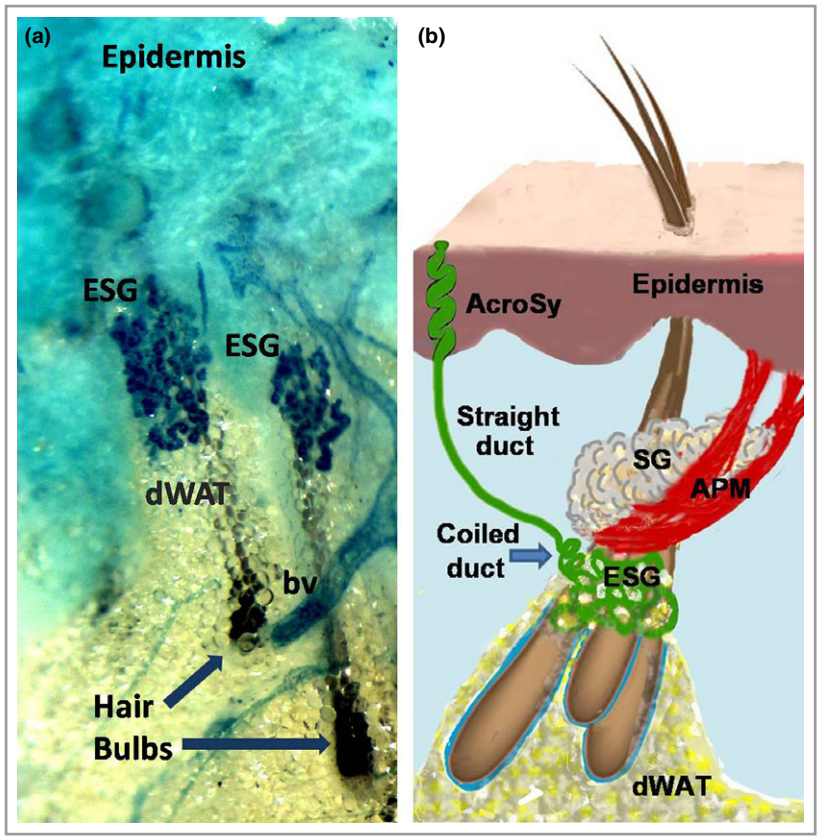

Fig 2. (a) This 60-μm-thick section of scalp skin stained with the methylene blue vital dye clearly shows the association between eccrine sweat glands (ESG) and their follicular units. ESGs can be identified at the tip of the dermal white adipose tissue (dWAT) that display the typical 'cone-shaped' morphology, being the upper part of the gland at the dermohypodermal interface. Blood vessels (bv) are also stained. (b) On this diagram the trajectory of the hair follicle-associated ESG is demonstrated from the intraepidermal acrosyringium (AcroSy) to a perifollicular area, embedded in a cone-shaped area of adipose tissue (dWAT) located below the arrector pili muscle (APM) and the sebaceous gland (SG).

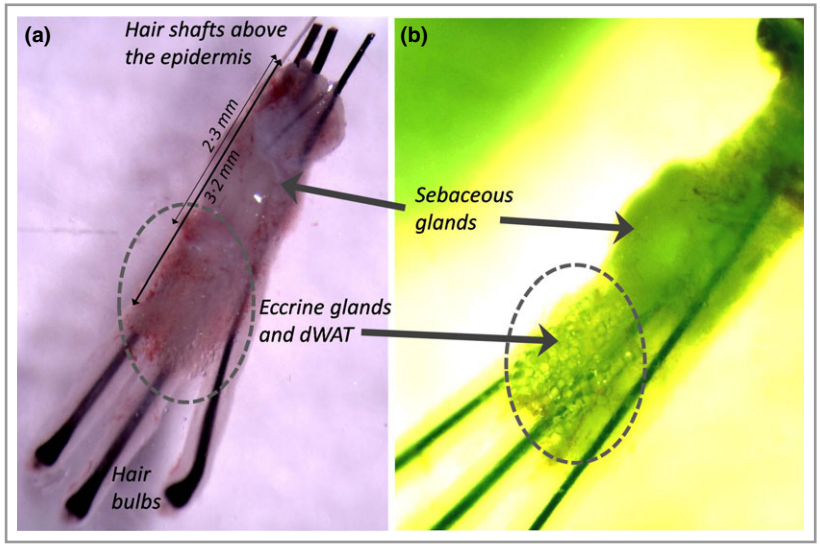

Fig 3. (a, b) Two separate follicular unit extractions from the scalp are observed containing three hair follicles and sebaceous glands that are easily identified as polilobulated, smooth-surface masses. Below the sebaceous glands an area with 'microbubbles' or foamy appearance can be identified. When observed microscopically this area corresponded to eccrine sweat glands surrounded by dermal white adipose tissue (dWAT).

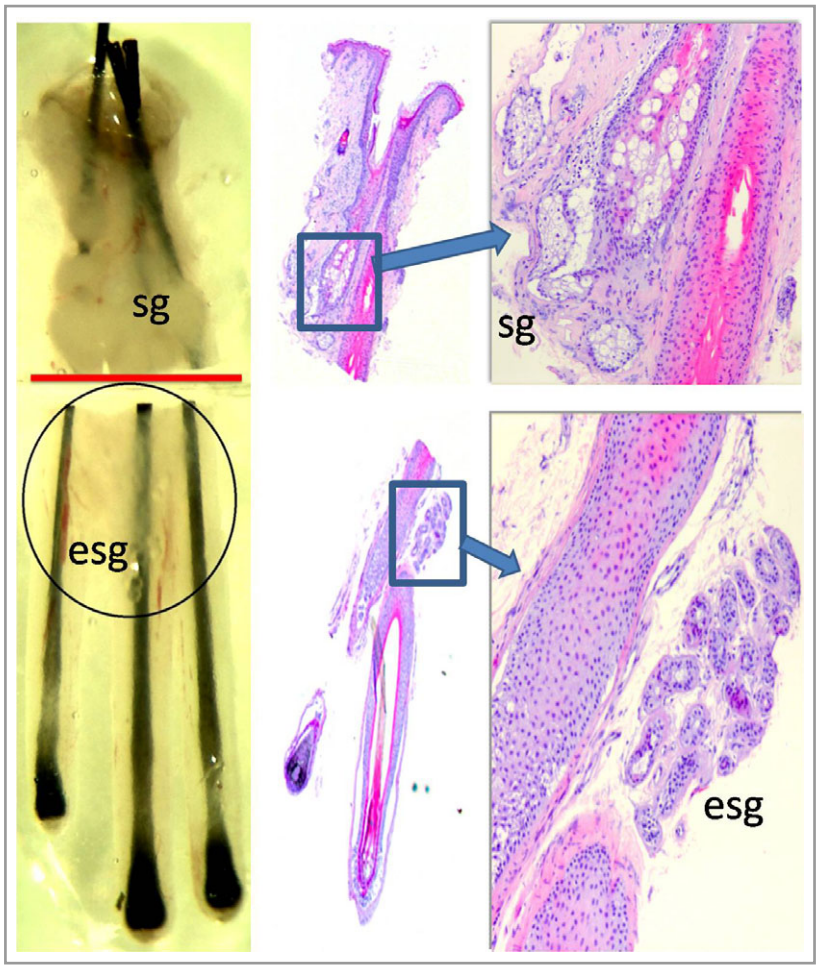

Fig 4. A follicular unit was bisected below the sebaceous gland (sg). When the two fragments were microscopically observed it could be confirmed that the sebaceous gland was in the upper fragment and that the tissue remaining attached to the follicular unit below the sebaceous gland is an eccrine sweat gland (esg).

More than one ESG duct could be observed leading to ESGs of the same FU (Fig. 5B), a finding that could explain the previous suggestions that ESGs ducts outnumber the HFs.[10,13]

The computer-assisted 3D reconstruction and its analysis from different angles of view clearly demonstrate the presence of HF-associated ESGs in healthy human scalp skin (Fig. 5B and Video S1; see Supporting Information).

Discussion

In this study we confirm our hypothesis[13] that the association of the secretory portion of ESGs with the PSU is a common finding, at least in human scalp skin, and document that the ESG coil lies in close proximity to the progenitor-cell-rich sub-bulge region of the HF epithelium[15–17] and its surrounding connective tissue sheath. Given the high density of ESGs in human scalp skin, one may wonder whether it is a mere consequence of tighter skin appendage packaging and spacing meaning that many HFs are in close proximity to ESGs. However, it is noticeable that the secretory part of the ESGs tends to be included in the FU complex itself below the attachment of the APM (Figs 2a and 5Ac), thus leaving the interfollicular areas almost free of ESGs. Also, the physical association of both components of the secretory segment of the ESG (i.e. the coiled duct and the secretory gland) is so fixed that it is preserved even in FUs harvested with a very narrow 1-mm biopsy punch; they remain attached to the HFs when they are pulled up and extracted.

Furthermore, in this study we show that the unique skin-resident adipocyte population referred to as dWAT[18] appears in clusters known as 'dermal cones' at the dermohypodermal interface around the PSUs and provides an environment into which the acini and the terminal ducts of the ESGs are embedded. To the best of our knowledge, the presence of ESGs in this special, cone-shaped part of the dWAT that invaginates the reticular dermis has not been reported previously. This may have been overlooked as dWAT has been explored mainly in mice,[19] where no HF-associated ESGs are present. However, it is noteworthy that ESGs share functional attributes[20] with the dWAT, such as thermoregulation[20] and antibacterial defence.[21]

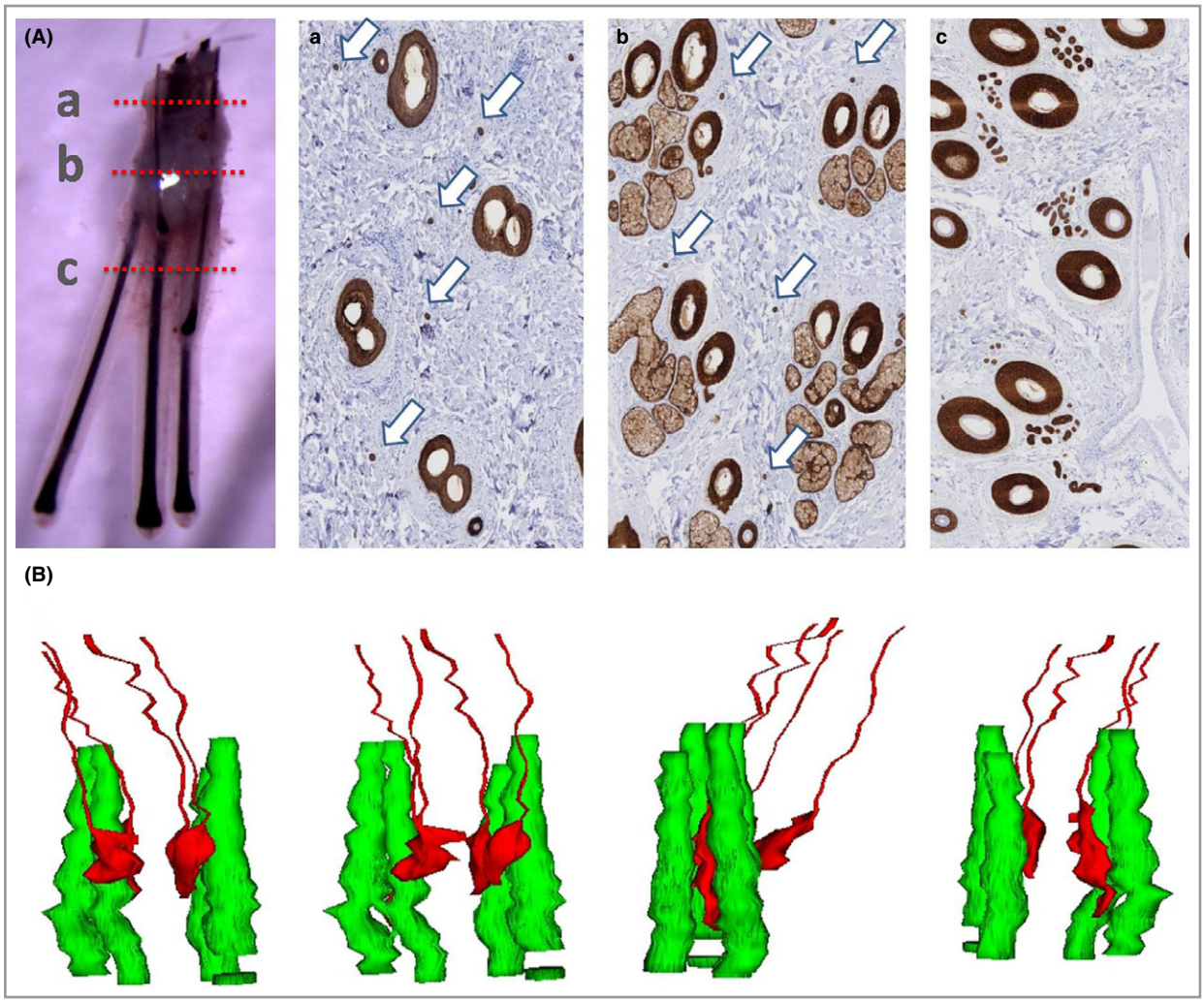

Fig 5. (A) A follicular unit from human scalp tissue represents the levels at which transverse sections of the skin immunostained with the pankeratin AE1/AE3 antibody were obtained: (a) at the isthmus, (b) at the sebaceous gland and (c) below the sebaceous gland. In the transverse section (a) the eccrine ducts (highlighted by open arrows) show a clear interfollicular disposition. In the transverse section (b), the eccrine sweat gland ducts still appear to be localized in the interfollicular area (arrows) but are closer to the follicular units. In section (c) eccrine sweat gland acini are included within the FUs. (B) Using multiple sections like those represented in (A), a computer-assisted 3D reconstruction could be built. Two follicular units with ESGs attached are represented at different angles of view. Two eccrine sweat ducts are directed towards each follicular unit.

Interestingly, some older reports already made passing reference to the proximity between the HF and ESG,[22,23] yet this was not followed up until recently.[13] This is probably because the conventional planar, vertically oriented sections of human skin fail to reveal the complex volumetric structures that interact in a 3D space, as commented on by Hermann Pinkus: 'The solution is not found in the fixed and dead microtome sections of our laboratories. We must think and work in the living three dimensions of bodily structure'.[24]

In fact, the proposed adnexal skin unit that we describe here is perfectly in line with the historical 'skin unit' or 'hair field' concepts envisioned by early skin morphologists.[25,26] The concept also sits well with the established concept that HFs are arranged in functionally relevant units (FUs)[27] that share a 'muscular unit', the APM,[28,29] and share innervation from a common sympathetic nerve branch that during its trajectory in the dermis is divided to produce nerve bundles that innervate the sebaceous gland, APM, HF, arterioles and epidermis.[23]

Obviously, ESGs are not associated with HFs in hairless regions of the integument, namely in palmoplantar skin. However, it is important to note that palmoplantar and scalp skin ESGs, for example, differ both embryologically and functionally,[30,31] so one should not simplistically extrapolate from one type of ESG to another. The first category of ESGs is present on friction surfaces of the palmoplantar skin of many mammalian orders, notably in mice and all primate species, including humans. These ESGs develop at about human

gestational month 3·5, 2 months earlier than elsewhere, suggesting that these represent more archaic ESGs.[30] It is believed that their main function is to increase surface friction by moistening keratin.[31] Instead, ESGs in hairy human skin, which start to develop much later (at the fifth month of gestation),[31] represent a distinct, evolutionarily younger ESG category, which functions primarily to cool the body by sweating (further discussion in Appendix S1; see Supporting Information).[31]

The reason why 20% of the FUs analysed in this study have no associated ESGs could be that we have searched for them with a simple stereomicroscopic method, and it is quite possible that a more refined 3D analytical method would reveal an even higher percentage. However, there are indeed some non-HF-associated ESGs in human scalp skin, just as in palmoplantar skin, which may be functionally similar to the latter, or that have survived after the originally associated FU has atrophied.

It is conceivable that the common microenvironment that ESGs, HFs and dWAT share represents a niche that is of major importance for skin regeneration, which complements the known regenerative functions of the HF connective tissue sheath and its progenitor cell population.[32–38] The regenerative capacity of epithelial cells from the ESG secretory coil is well known,[39,40] and ESGs are major players in the re-epithelialization of human wounds.[6,41] Moreover, label-retaining epithelial progenitor cells in ESGs have features in common with HF epithelial stem cells expressing keratin 15,[42,43] and ESG epithelial cells can not only regenerate the ESG itself but can also undergo differentiation into epidermal keratinocytes so as to reconstruct an epidermis-like structure *in vitro*.[11,44,45]

Intriguingly, ESG cells display high plasticity and can reconstitute HFs in experimental conditions.[43] In addition, the stroma of ESGs is the main source and site of pluripotent nestin-positive stem cells in human skin, which can differentiate into cells of all lineages.[8,9] Complementing this, the dWAT is a rich source of adipose tissue-derived stem cells that can promote wound healing and skin regeneration.[46–49] Therefore, the agglomeration of HFs, ESGs and dWAT within a shared skin microenvironment may provide a tissue niche to facilitate skin regeneration. On this basis, future research should investigate how each component influences the other in a common niche such as by paracrine interactions, especially of certain shared mediators, such as thyroid hormones, prolactin or substance P.[50–52] Experimentally, the functional interconnections between the ESG, HF and dWAT may best be interrogated in organ-cultured intact human scalp FU.[53]

It is of practical importance that the easily discernible 'microbubbles' that we show here in harvested FUs represent a new macroscopically distinctive hallmark that permits the identification of both ESGs and dWAT. ESGs are not visible in unstained FUs, yet their presence and location can be inferred from where the microbubbles are visible. Thus, the new FU hallmark that we describe here greatly facilitates macroscopic ESG identification. Also, the relatively easy accessibility of FUs could represent a new source for obtaining unique stem cells with multilineage differentiation potential and with promising perspectives for clinical therapies.

The proposed HF–ESG association may also be clinically relevant, as it may help us to understand better the sweat gland alterations that have been reported in selected forms of alopecia,[54,55] including excessive localized hyperhidrosis in patients with frontal fibrosing alopecia[56] and the increased number and volume of ESGs in androgenetic alopecia scalp skin.[57] Also, future research into wound healing and selected skin diseases (involving one of the components discussed here) should routinely consider the adnexal skin unit concept.

In conclusion, the existence of HF-associated ESGs establishes a new model of functional human skin anatomy in which ESGs are intimately associated with the HF and the dWAT to form a common homeostatic environment. The challenge now is to dissect functionally how each component of this newly recognized superstructure of human skin cooperates with and influences the others under physiological conditions, during skin wounding and regeneration and in selected skin diseases.

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Supporting Information

Additional Supporting Information may be found in the online version of this article at the publisher's website:

Appendix S1 Supplementary discussion and references.

Fig S1. The microbubble-containing tissue that remained attached below the sebaceous gland was selectively stained with India ink. The haematoxylin and eosin-stained section of tissue demonstrates that this area corresponds to an eccrine sweat gland embedded into adipose tissue.

Video S1 A three-dimensional video was obtained using multiple horizontal sections immunostained with the pankeratin AE1/AE3 antibody and processed with Reconstruct version 1·1·0·0 software.