An efficient method for eccrine gland isolation from human scalp

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An efficient method for eccrine gland isolation from human scalp

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An efficient method for eccrine gland isolation from human scalp

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Supplementary Material: Text (348 words), Figures (2), Video (1)

Keywords: Eccrine glands, sweat glands, hair follicle, hair transplantation, hair graft, follicular unit, sweat, neutral red, methylene blue

Abstract:

We describe a simple and efficient method to isolate eccrine sweat glands from the human scalp. This method is inspired by the hair graft harvesting method used in hair transplantation. Based on the recently described anatomical relationship between the scalp hair follicle and the eccrine gland, we have found that scalp follicular unit grafts are an excellent eccrine gland isolation source, especially for the coiled component. In order to make the gland visible for stereoscopic microdissection, the follicular units need to be previously stained with a vital dye like Methylene Blue or Neutral Red. The simplicity and efficiency of this isolation method should encourage further research into human eccrine sweat gland function which has always been hindered by the difficulty of gland isolation.

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Background

Eccrine glands (EGs) are cutaneous appendages that produce sweat and act as our primary source of cooling. Besides their critical role in thermoregulation, they also play a less understood yet important role in cutaneous wound healing (1-4). Research into human eccrine sweat gland function has been greatly hindered by problems of gland isolation (supplementary text). Obtaining full intact EGs by stereoscopic microdissection of human skin is a very difficult, laborious and low yield-giving technique (5-11). Although other isolation methods such as enzymatic collagenase digestion do yield a larger number of glands, exposure to enzymatic digestion may be potentially damaging for physiological functional studies (12-15). In this article, we describe an efficient method of isolating human eccrine sweat glands from follicular unit grafts harvested from the scalp. The idea of harvesting EGs from the scalp arose originally after our reported observation of the striking and consistent anatomical association between eccrine coils and hair follicles (HF) (16).

Question Addressed

Given the intimate association between the EG and HF, can eccrine coils be successfully isolated from human follicular unit (FU) hair transplant grafts?

Experimental Design

Our method of EG isolation is based on two premises: 1) due to the reported anatomic association between EGs and scalp HFs, we thought that an excellent and readily available source to obtain EGs was from FU grafts harvested in hair transplant

procedures, and 2) in order to isolate the EGs by stereoscopic microdissection, a supravital dye could be used to make them visible under a stereomicroscope. Different supravital dyes to highlight the EGs were tried, including Methylene Blue (MB), and Neutral Red (NR) (6, 17,18). All FUs were donated by patients undergoing hair transplant surgery for androgenetic alopecia after giving written informed consent. As a reminder, the FU is the prime element used in modern hair transplantation (19). An FU is a histological structure composed of 1 to 4 terminal hair follicles along with their sebaceous glands and arrector pili muscles (20). FUs can be obtained in hair transplantation in two ways: 1) by directly excising individual FUs using small round punches (0.8 to 1.00 mm in diameter), a technique known as FUE (Follicular Unit Extraction) (21), or 2) by stereomicroscope dissection of small slivers sectioned from a donor strip (strip harvesting technique) . In the FUE technique, the punch is introduced to a depth of around 3 mm, and the FU is then released from the subcutaneous tissue with fine tip forceps ([supplementary video](#)) ([Fig 1](#)). It is important to have the skill to insert the punch following the hair shaft angle in order to avoid hair follicle transection.

Results

MB and NR gave a very clear delineation of the EG, providing a distinct blue and red coloring of the EG, respectively, easily visible against the background of the dermal collagen. We found that immersing the FU in a few drops of MB at a concentration of 0.02% or in NR at a concentration of 0.2% in physiologic saline for 10-15 minutes was sufficient to highlight the EGs ([Figure 2 & Supp. Fig 2](#)).

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EG isolation can be accomplished using either of the two different FU graft harvesting methods: strip harvesting and FUE. Using the strip harvesting technique, we observed that the small vertical slivers in which the long strip is divided - of 1 to 2 FU thickness - contain numerous EGs that can be easily identified by MB or NR staining and later microdissected (Fig. 2 A,B).

In our experience, EG isolation was even easier when using FU grafts harvested with FUE punches, because the small punches used to excise the FUs, left minimal dermal surrounding tissue, making EG coil dissection faster since the coil was not attached to dermal fibers (Fig. 2 C,D). Anatomically, the EGs are always embedded in adipocytes (dermal fat tissue) and located at a depth of 2 to 3.5 mm below the epidermal surface of human scalp, at the inferior portion of anagen terminal hair follicles, and always below the sebaceous gland and arrector pili muscle (Suppl Fig. 1). We noted that the smaller the caliber of the punch used in FUE, the higher the risk of transecting and damaging the EG coil. For this reason, we believe that the use of 1 mm diameter punches is ideal for this purpose. In addition, the ductal excretory portion cannot be isolated with this method because at some point the duct is transected by the punch as depicted by the schematic in figure 1.

Although the main goal of this study is to describe a rapid and efficient source for EG isolation, *ex vivo* culture of isolated EGs in a mixture of supplemented Williams E and F12 media, demonstrated that they are still viable after 6 days as shown by Neutral Red uptake and Calcein AM (2µM) (ThermoFisher Scientific), a cell-permeant dye that in live cells is converted to green-fluorescent calcein (Supp. Fig. 2).

Conclusion

With the emerging discovery of the importance of the EG in roles other than sweating (including, amongst others, wound healing), an EG isolation method is required that is simple, efficient, and that can also be used with living tissue to maintain its morphologic integrity.

Researchers should consider scalp FU grafts obtained from hair transplant procedures as an efficient source for isolating human EGs. Moreover, once the eccrine glands have been dissected from the FUs, the hair follicle grafts could still be used for hair transplantation, so no human tissue is wasted.

Isolation of human EGs *ex vivo* that maintain their morphological integrity and function can also be an invaluable source for the cosmetic industry interested in studying the sweat response to pharmacological products in healthy as well as in pathologic disorders (e.g. anhidrosis, hypo and hyperhidrosis). As a further fascinating challenge, the possibility exists of investigating the clinical outcome after transplanting exclusively EGs in special clinical situations, for example to extensive deep-burn survivors whose scarring skin covered with skin grafts is devoid of sweat glands and suffers from heat intolerance (22-24).

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Figure Legends

Figure 1. Schematic of eccrine gland isolation technique using an FUE punch.

This figure illustrates the technical procedure of eccrine gland isolation from the scalp follicular unit with a circular micro-punch (FUE technique). The left drawing depicts the anatomy of a scalp follicular unit (FU), showing the location of the eccrine coil in relation to the anagen terminal hair follicles, sebaceous glands and the arrector pili muscles. Note that most human scalp FUs contain 2 or 3 terminal hair follicles. The eccrine coil is embedded in adipocytes (dermal fat tissue). The arrector pili muscles that emerge from each of the follicles that form the FU join together forming a single muscular bundle. The middle drawing shows how the punch needs to be introduced into the scalp skin with the angle of the punch parallel to the hair shaft direction in order to avoid FU transection. The penetration of the punch to a depth of 3-4 mm is sufficient with most patients to release the FU from the surrounding dermal collagen attachments and allow it to then be easily removed with fine tip forceps (drawing on the right).

Figure 2. Methylene Blue allows easy identification of human eccrine glands in scalp

follicular units. A typical thin vertical sliver of scalp skin dissected under the stereomicroscope during a strip harvesting hair transplant procedure unstained (A) and stained with Methylene Blue (B). Note the position of the eccrine coils, in the deep dermis, approximately 2-3.5 mm beneath the epidermis and embedded in adipocytes. A three-hair follicular unit (harvested with a 1 mm punch) stained with Methylene Blue with two eccrine coils between the hair follicles (C) and the eccrine coils after dissection from the follicles (D).

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Only

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3 **Supplementary text:**
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7 ***Methods of eccrine gland isolation:***
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10 Sweat gland research is still rather slow in comparison with other fields, with one of
11 the main reasons being the difficulties in isolating it from human skin. Microdissection
12 has been tried, but this is an extremely difficult technique which gives a low yield (5-
13 11). Sato (7) isolated sweat glands from 1 cm x 0.5 cm ellipse skin from the forearms,
14 under a stereomicroscope, by gently teasing away periglandular collagen fibers with
15 sharp forceps. The method is very difficult and time-consuming and has a low
16 efficiency. Lee et al (10) and Brayden et al (20) described a better way to isolate
17 human sweat glands by chopping skin samples with sharp scissors. The principle was
18 that sweat glands “pop out” from surrounding collagen and fat due to shearing forces
19 created by the chopping.
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22 Another way to isolate glands is by enzymatic digestion of skin samples with
23 collagenase. This method involves overnight incubation of skin samples with
24 collagenase. It yields a larger number of glands (14-16), but the exposure of cells to
25 collagenase is potentially damaging.
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28 One of the difficulties encountered with EG isolation by microdissection is that
29 EGs, as opposed to sebaceous glands or hair follicles, are not visible under the
30 stereomicroscope, which makes dissection impossible. To circumvent this problem,
31 Wolfe (8) and Mangos (9) stained the gland prior to dissection with the supravital dye
32 Methylene Blue. This dye can be introduced in the skin without damaging the cells. It
33 has been used in the past to study eccrine sweat gland function by intradermal
34 injection and evaluation of the blue sweat droplets excreted at the ductal orifices (19).
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Another vital dye used to stain eccrine glands is Neutral Red (20). Neutral red is actively secreted into the lumen of the duct through organic ion pathways. According to Brayden (20), Neutral Red staining allowed on average 52% more sweat glands to be isolated from the chopped suspension of human skin. The Neutral Red staining method has been shown to be non-toxic at low concentration and the stained glands grow in culture just as successfully as controls.

Supplementary Figures:

Supplementary Figure 1.

Eccrine glands are clearly visualised after follicular unit grafts are stained with Neutral Red and Methylene Blue

Follicular units harvested from scalp skin with a 1mm punch and stained for 10-15 minutes with (a) Neutral Red (0.2%) and (b) Methylene blue (0.02%) provides a very clear intravital identification of the eccrine glands (arrows), which are normally not visible under the stereomicroscope.

Supplementary Figure 2

Eccrine glands are still viable after 6 days of ex vivo culture

After 6 days of culture in a mixture of supplemented Williams E and F12 media, eccrine glands were stained with two markers to assess their viability: Neutral Red (a) which

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stains lysosomes of live cells, and (b) Calcein AM, a cell-permeant dye which in live cells the non-fluorescent calcein AM is converted to green-fluorescent calcein.

Supplementary Video:

Follicular unit extraction: Surgical technique using a motorized 1 mm punch for harvesting follicular units from the scalp. These FUs can be stained with Neutral Red or Methylene Blue for eccrine gland identification under the stereomicroscope.

For Review Only

Eccrine glands are clearly visualised after follicular unit grafts are stained with Neutral Red and Methylene Blue

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Eccrine glands are still viable after 6 days of ex vivo culture

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Review Only

Author's Response to Decision Letter for (EXD-17-0449)

An efficient method for eccrine gland isolation from human scalp

Dear Professor Luger,

We would like to thank the reviewers for their kind comments and helpful insight/suggestions. We have addressed the reviewers' questions as following:

Reviewer 1:

1. How long can the authors culture the eccrine glands after they isolate eccrine glands?

We have shown that eccrine glands maintain viability after 6 days of culture as assessed by uptake of the cell viability markers Neutral Red and Calcein AM. We have added this information to the supplementary text of the manuscript and supplementary figure 2.

2. What kind of culture medium should be used for eccrine gland organ culture?

A mixture of supplemented Williams E and F12 media was used to culture eccrine glands. This has been added to the main text.

3. Could the authors more specify the possible further experiments which can be performed with this isolation technique?

Further experiments could be to utilise this method to test the functionality of eccrine gland i.e. by stimulating with known sweat inducers such as Acetylcholine, as done in a previous study by Sato et al. in the 70s (Sato et al. 1973, American Journal of Physiology) using eccrine glands from monkeys and more recently in 3D organotypic models of eccrine glands (Klaka et al. 2017, Plos One). We could also do the opposite, i.e. to inhibit the sweat production by treating with for example botulinum toxin.

Reviewer: 2

Suggestions for Authors

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This letter by Jimenez and colleagues describes a method for the isolation of eccrine sweat glands from the human scalp. The method is actually not new, as it consists essentially of already established procedures (punches or the strip harvesting technique). Nevertheless, considering the increased interest in eccrine gland research and the lack of simple and effective methods for their isolation, this is an important study. What a terrific movie!

1) The authors repeatedly mention what seems to be a limitation of the method: the fact that only the coiled compartment of the gland can be isolated, while the ductal secretory portion gets lost. However, what does this limitation exactly mean? What kind of basic science or translational studies are handicapped by the loss of this particular anatomical part of the gland?

From a functional point of view, since the coiled gland is the main component of the eccrine gland where the sweat is produced, the loss of the 'straight' ductal portion does not pose a great limitation to further investigation in the context of sweat production. We feel that the main limitation of transecting the sweat ducts would be in studying the role of sweat glands in skin wound healing, since it has been shown that there is a rapid proliferation of the sweat duct epithelium after superficial injuries (Rittié et al. Am J Pathol 2013).

2) Figure identification and labelling need to be significantly improved. For instance, on page 5, line 39, Fig. 1c,d are indicated, while there are not such labels in the figure or in its legend. Are there 3 or 4 suppl. figures? Suppl. Figures need better legends with improved description.

Thank you for highlighting this, it has been corrected with the previous supplementary figures 1 & 2 combined into one figure and more descriptive legends have been added.