SUPPLEMENTARY INFORMATION

SUPPLEMENTARY NOTE

Supplementary Note | Extended discussion of methodology development and troubleshooting

For skin organoid induction, we made substantial modifications to our previously published inner ear organoid protocol[1]. In the Koehler Lab (Indiana University School of Medicine and Boston Children's Hospital/Harvard Medical School), we primarily used two different human pluripotent stem cell (PSC) lines—WA25 embryonic stem cells (ESCs) and *Desmoplakin-GFP* (*DSP-GFP*) induced PSC (iPSCs)—to optimize the protocol. Then, the optimized protocol was validated in the Heller Lab (Stanford University) using a different cell line, the WA01 (H1) hESC line. We aimed to establish a protocol that was reliable and applicable to multiple PSC lines. Due to the 3D format and the long duration of the cultures, extensive quantification of cell aggregate morphology and cellular composition was not practical for every treatment regime tested. Therefore, we often used qualitative metrics (e.g. cell aggregate appearance) to discontinue culture conditions at early developmental timepoints, which we judged to be less likely to produce hair-bearing skin organoids. For conditions cultured over 70 days, we have provided the percentage of organoids that displayed hair follicles as a key metric of successful skin organoid generation (see **Supplementary Table 1a**). Below is a brief summary of our optimization approach:

Basal medium optimization: We first sought to substitute an off-the-shelf medium for the Chemically Defined Medium (CDM) used in our inner ear organoid induction protocol. Because CDM was manually prepared for each experiment, we found that pipetting errors, reagent stability, and batch effects were sources of experimental variability. We substituted out CDM for the commercially available Essential 6 (E6) medium (Invitrogen/Gibco) because it is stable, fully defined, and convenient for use. Moreover, E6 medium has been shown to promote highly efficient ectoderm differentiation from PSCs[1]. After switching to E6 medium, however, we noted subtle differences in organoid morphology. Specifically, neural epithelia were more prevalent. Thus, we modified the small molecule and protein treatments for proper skin organoid induction.

Treatment regime optimization: For efficient surface ectoderm formation, the proper concentration and timing of bone morphogenic protein 4 (hereafter, BMP) is critical. In our hands, each PSC line has a different BMP concentration requirement for surface ectoderm induction. This phenomenon is potentially due to varying levels of endogenous BMP between cell lines. Therefore, to optimize surface ectoderm formation, we focused on BMP concentration and timing during the first 0 - 4 days of differentiation. We then re-optimized the timing of LDN and FGF treatment. Below is a brief summary of these experiments:

> *BMP concentration/timing optimization*: For *DSP-GFP* cell line, 2.5 – 5 ng/ml of BMP treatment was suitable for skin organoid induction; while 10 ng/ml was also suitable, the outcome was similar to 5 ng/ml BMP. For WA25 cell line, 0 – 2.5 ng/ml of BMP treatment was suitable for skin organoid induction, while 5 ng/ml of BMP was excessive. Thus, we selected 2.5 ng/ml as our universal BMP concentration.

For *DSP-GFP* cells, treatment of BMP was required from the starting day (*day 0*) of differentiation. BMP treatment beginning on *day 1* partially produced skin organoids, and the treatment on *day 2* or later did not induce skin organoids. For WA25 cell line, BMP treatment timing was more flexible and could be performed any time between *day 0* and *day 1*.

For optimized differentiation of both cell lines, we selected 2.5 ng/ml BMP on the starting day (*day 0*) of differentiation, in addition to the other previously established medium ingredients: E6 basal medium containing 2% Matrigel, 10 μM SB, 4 ng/ml FGF, and 2.5 ng/ml BMP.

LDN/FGF treatment timing optimization: To optimize induction of CNCCs, we adjusted the timing of BMP inhibition (LDN treatment) in combination with FGF treatment.

We first examined the timing of LDN/FGF treatment. We found that the required treatment timing varied between *day 3* and *day 4*, depending on the BMP treatment conditions. For WA25 cell line, when BMP was not added, LDN/FGF was required on *day 4* to induce skin organoids. When BMP was added on *day 0*, LDN/FGF was required on *day 3*. In *DSP-GFP* culture, with *day 0* BMP treatment, LDN/FGF treatment on either *day 3* or *day 4* induced skin organoid formation, but *day 3* treatment was more efficient compared to *day 4* treatment (see **Supplementary Table 1b** for results).

We next tested whether FGF treatment would be necessary for efficient skin organoid induction. In WA25 cell line cultures without BMP, LDN treatment on *day 4* induced skin organoids in some cultures, but the efficiency was highly variable from 0 to 66.7% throughout five independent experiments. When LDN was treated in combination with FGF, regardless of treatment timing between *days 4-8*, we consistently observed hair-bearing skin organoids induction: 66.6 ± 11.2% of organoids from five independent experiments (n = 65 organoids). Likewise, in *DSP-GFP* cell line cultures with *day 0* BMP treatment, LDN and FGF co-treatment on *day 3* was more efficient than LDN treatment alone. (see **Supplementary Table 1c** for results).

We concluded the optimal LDN/FGF treatment for both cell lines was 200 nM LDN and 50 ng/ml FGF co-treatment on *day 3* of differentiation.

Matrigel usage optimization: Lastly, we compared Matrigel embedding versus floating culture on *day 12* of differentiation. In our pre-optimized CDM protocol, we embedded individual aggregates in Matrigel (~20 μl per aggregate) droplets as in our inner ear induction protocol. This approach is time consuming and expends large volumes of Matrigel. To overcome these drawbacks, we tested culturing individual aggregates in a floating culture containing 1% Matrigel. We found that 1% Matrigel dissolved in the Organoid Maturation Medium (OMM) was sufficient to support hair-bearing skin organoid induction. Notably, for this study, we did not test whether skin organoids could be produced in the complete absence of Matrigel at all steps of the protocol.

Excluded cell lines: Two cell lines were excluded from our dataset due to a lack of proper quantitation. We tested the protocol on the popular WA09 (H9) hESC cell line and the mND2-0 hiPSC line (both supplied by WiCell). We found that the WA09 cell line displayed poor induction of surface ectoderm cells and excess production of neural crest-like cells—consistent with previous reports that this line has a neuroectoderm bias. We did not optimize the protocol

for use with WA09 cells, nor did we carry out long-term culture to determine whether this line could produce hair-bearing organoids. The mND2-0 cell line performed similarly to the other cell lines in this study and generated hair-bearing skin organoids; however, the number of long-term cultured (>70 days) specimens generated (n = 5) was not satisfactory for inclusion in this study.

SUPPLEMENTARY DISCUSSION

Supplementary Discussion | What type of hair grows on our skin organoids?

With our current data, it is uncertain whether skin organoids represent a distinct anatomical location. The gene expression at day-6 of differentiation in our skin organoids suggests that the organoids are patterned broadly to various anterior-posterior levels in the cranial region. Gene expression analysis at day-29 hints at a pharyngeal arch 1 identity. However, these findings are not definitive and need further confirmation with scRNA-seq analysis on additional biological replicates at additional timepoints.

One way to determine positional identity may be to compare features of organoid-derived hair follicles to site-specific features of hairs in the body. The medullar layer is a prominent feature of terminal beard hairs and, if present, would bolster evidence that skin organoids represent mandibular facial skin. We performed immunostaining for several medulla markers reported by *Langbein et al.* 2010 - KRT5, 7, 14, 16, 17, and 75 - on hair follicles of skin organoids and foetal tissues (both forehead and cheek), but none of the hair follicles presented those markers in the innermost layer of the follicle (data not shown)[2]. The human foetus is initially covered by lanugo hair, which sheds before or shortly after birth to make way for vellus and terminal hairs. Terminal hairs are the only human hairs that contain a medulla layer. Thus, our data suggest that skin organoids produce lanugo-like hairs rather than vellus or terminal hairs, which do not typically develop until after birth. We suspect that additional optimization of the culture system, incorporating various hormones (e.g. testosterone), could transition organoid hairs to these more mature phenotypes.

SUPPLEMENTARY REFERENCE

1. Koehler, K. R. *et al.* Generation of inner ear organoids containing functional hair cells from human pluripotent stem cells. *Nat Biotechnol* **35**, 583–589 (2017).

2. Langbein, L., Yoshida, H., Praetzel-Wunder, S., Parry, D. A. & Schweizer, J. The keratins of the human beard hair medulla: the riddle in the middle. *J Investigative Dermatology* **130**, 55–73 (2010).