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***Dermocosmetics***”

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## 1. Introduction

The skin, the body's largest organ, plays a crucial role as a protective barrier between internal systems and the external environment. It is composed of three main layers: the epidermis, primarily made of keratinocytes that undergo keratinization to form the stratum corneum; the dermis, rich in fibroblasts that produce collagen and elastin to provide strength and elasticity; and the hypodermis, which is largely composed of adipocytes that insulate and cushion the body [1]. In recent decades, growing insights into skin physiology and the emergence of advanced testing technologies have revolutionized the cosmetic field [2]. This has led to the development of innovative active ingredients and delivery systems based on scientifically validated mechanisms of action, as well as more stringent guidelines for efficacy and safety assessments. These advances have shifted the concept of cosmetics from simple beautifying agents to dermocosmetics, topical formulations that merge dermatological function with cosmetic appeal, offering therapeutic benefits for various skin conditions [3].

Despite these advancements, research on skin biology and the safety evaluation of dermocosmetic products has traditionally depended on animal models, which are often costly, ethically problematic, and not always reflective of human skin responses [4]. As a result, the European Union implemented a ban on animal testing for cosmetics in 2013, and the FDA announced plans to reduce animal testing requirements by 2030, further supporting the global transition to alternative methods [5], [6]. In this context, the generation of in vitro skin models has become essential and many option have been developed. Widely used Reconstructed Human Epidermis (RHE) closely mimic the layered architecture and barrier properties of human skin, and are validated for regulatory irritation and corrosion testing in the guidelines [7], [8]. Skin equivalents, which are composed by layered keratinocytes on dermal elements like fibroblasts embedded in a collagen matrix, reflect the dermal and epidermis layers and allow for more accurate assessments of dermal penetration and inflammatory responses [9], [10]. Although these models effectively mimic the structural organization of the skin, they still lack its full complexity, as they do not have appendages like hair follicles and sweat glands, as well as diverse cell populations such as immune cells [11], [12]. Among this variety of 3D models, skin organoids are emerging as a cutting-edge tool in skin research, offering a novel approach to studying skin biology. Derived from pluripotent stem cells, such as induced pluripotent or embryonic stem cells, these three-dimensional structures self-organize to form simplified skin equivalents, often comprising both epidermal and dermal layers. Moreover, these models can also generate appendages like hair follicles, sebaceous glands, sensory neurons, adipocytes and melanocytes [13], [14], [15], [16]. As these models are derived from induced pluripotent stem cells, they offer a significant advantage over traditional explants, biopsies, or primary cells due to their ability to provide an infinite and reproducible source of material. Unlike conventional methods that rely on limited tissue samples, iPSC-derived organoids can be generated from individuals of diverse genders and ethnic backgrounds, enabling more personalized and representative models [17]. This unique feature holds considerable promise for advancing both basic research and cosmetic testing, providing a powerful tool for evaluating skin-related treatments. The aim of this study was to assess the utility of

hair-bearing skin organoids as a model for dermocosmetic testing. Specifically, we focused on evaluating the key claims in the dermocosmetic field, including toxicity, anti-aging effects, hair growth and anti-inflammatory properties. To this end, we treated our models with reference molecules, including isopropanol, hexyl salicylate, and heptanal, as outlined in OECD 439 guidelines [7]. Additionally, we investigated the known antioxidant and collagen-promoting effects of ascorbic acid (Vitamin C) [18], [19], [20]. The inflammatory response to lipopolysaccharide (LPS) and its modulation by dexamethasone were also evaluated [21]. Finally, given the presence of hair follicles in our skin organoids, we explored their potential response to Minoxidil, a widely used hair growth treatment [22]. This study represents the first evaluation of the potential of hair-bearing skin organoids in the context of dermocosmetic testing, offering insights into their applicability for assessing skin and hair-related therapies.

## 2. Materials and Methods

### 2.1 Organoids generation

Organoids were generated from human induced pluripotent stem cells (hiPSC) using a proprietary protocol developed in-house.

### 2.2 Treatments conditions

To assess the functional responsiveness of human skin organoids, a range of compounds was applied to model distinct physiological and pathological conditions. Mature skin organoids (Day 120 or older) were treated under controlled culture conditions with known reference molecules. For the assessment of skin irritation, the OECD Test Guideline 439 was applied using isopropanol (Sigma) , hexyl salicylate (Sigma), and heptanal (Sigma) as test compounds. Phosphate-buffered saline (PBS) served as a negative control, while 5% sodium dodecyl sulfate (SDS) was used as a positive control.To investigate extracellular matrix gene regulation, organoids were exposed to ascorbic acid (Sigma) at 1 µM and 100 µM concentrations. Inflammatory responses were modeled through treatment with 10 µM LPS (eBioScience), and modulation of these responses was evaluated using 10 µM dexamethasone (Sigma), either co-administered or used as a pretreatment.To explore hair follicle activity and regeneration, organoids were treated for 14 days with minoxidil (Sigma) at 1 µM and 10 µM concentrations.

### 2.3 XTT assay

Skin organoids were transferred into 24-well plates and incubated with test compounds in accordance with the OECD Test Guideline 439. Cell viability was assessed using the CyQUANT™ XTT Cell Viability Assay (Invitrogen), following the manufacturer's instructions. Absorbance was measured at 450 nm, with a reference wavelength at 660 nm, using a Spark microplate reader (Tecan).

### 2.4 RNA extraction and quantitative PCR

Following treatment, organoids were transferred to RNAlater stabilization solution (Invitrogen) to preserve RNA integrity prior to extraction. Organoids were then washed in PBS and transferred to lysing tubes (Ozyme). A total of 700 µL of QIAzol Lysis Reagent (Qiagen) was added to each tube. Mechanical homogenization was performed using the Precellys Evolution homogenizer. RNA extraction was subsequently carried out using the RNeasy® Lipid Tissue Mini Kit (Qiagen) according to the manufacturer's instructions. Reverse transcription was performed using the High-Capacity cDNA Reverse Transcription Kit (Thermo Fisher Scientific).

### 2.5 Immunofluorescence on whole organoid

Skin organoids were incubated overnight at room temperature on a shaking platform in a permeabilization solution composed of 1% saponin, 2% bovine serum albumin (BSA), and phosphate-buffered saline (PBS). Primary antibodies anti-Krt17 (Invitrogen) and anti-Ki67 (Invitrogen) were diluted in the same permeabilization solution and incubated with the organoids for 3 days. After primary incubation, organoids were washed in permeabilization solution for 24 hours. Secondary antibodies were then applied for 48 hours, followed by an additional 48-hour wash. Following staining, organoids were washed with PBS and incubated overnight in RapiClear (Nikon) solution for tissue clearing. Imaging was performed using a SP8 Leica confocal microscope at 20× magnification and images were then analysed using Imariss software.

### 2.6 Statistical analysis

Statistical analysis was performed using Prism (GraphPad). A one-way ANOVA tested group differences, with significance set at $p < 0.05$ ($p < 0.05$,$p < 0.01$, $p < 0.001$). Post hoc tests (e.g.,Tukey) identified specific group differences. Data are presented as mean ± SEM.

## 3. Results

### 3.1 Comparative cytotoxicity assessment in skin organoids

To evaluate the responsiveness of our 3D skin organoid model to known skin irritants, we followed the OECD TG 439 protocol [7]. Sets of 5 mature organoids (from Day 100 onward) were exposed to reference compounds including isopropanol, hexyl salicylate, and heptanal. Phosphate-buffered saline (PBS) and 5% sodium dodecyl sulfate (SDS) were used as negative and positive controls, respectively. Organoids were incubated with the test substances for 15 minutes, followed by washing and a 48-hour post-treatment period before performing the XTT viability assay, in line with OECD guidelines to detect pronounced rather than transient cytotoxic effects. Absorbance readings were taken hourly from 1 to 6 hours (Fig. 1a), with the highest cytotoxicity observed for heptanal and 5% SDS. In contrast, isopropanol and hexyl salicylate induced only mild to negligible toxicity compared to the control. The most distinct viability differences were recorded at the 6-hour mark (Fig. 1b).

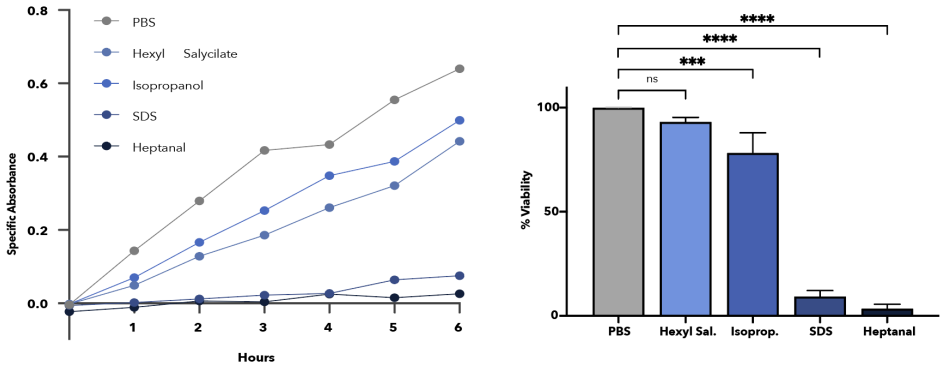



*Figure 1: Viability evaluation through XTT assay on skin organoids. (a) Viability of mature skin organoids (Day 100+) following 15-minute exposure to reference compounds: isopropanol, hexyl salicylate, heptanal, phosphate-buffered saline (PBS, negative control), and 5% sodium dodecyl sulfate (SDS, positive control). XTT-based viability assay was performed after a 48-hour post-treatment incubation. Absorbance readings were collected hourly from 1 to 6 hours post-XTT addition. (b) M*

| | Hexyl Salycilate | Isopropanol | SDS | Heptanal |
|---|---|---|---|---|
| OECD 439 | 95% (+/-7%) | 81% (+/- 15%) | 8% (+/- 6%) | 6% (+/-7%) |
| BioHive Toxicity | 93.17 % (+/- | 78.23 % (+/- | 9.3 % (+/- 2.9) | 3.5% (+/- 2.1) |

When compared with results from the standard Reconstructed Human Epidermis model outlined in OECD TG 439 (Table 1), our organoid data showed strong concordance, with skin organoids displaying reduced variability.

### 3.2 Vitamin C induces dose-dependent upregulation of extracellular matrix genes in Skin organoids

To assess the anti-aging potential of ascorbic acid (vitamin C) on skin organoids, we investigated its effect on the transcriptional regulation of key extracellular matrix components. Triplicate sets of mature skin organoids were treated with 1 µM and 100 µM concentrations of ascorbic acid for 48 hours. Total RNA was extracted and analyzed by quantitative PCR to measure the expression levels of *COL1A2*, *COL3A1*, *ELN*, and *MMP1* (Fig. 2).

Both *COL1A2* and *COL3A1* exhibited a dose-dependent increase in mRNA expression, with the 100 µM condition showing the most pronounced effect (Fig. 2a, c). *ELN* expression was significantly upregulated at 100 µM, while no significant change was observed at 1 µM compared to control (Fig. 2b). *MMP1* expression was slightly reduced in treated organoids, though this decrease was not statistically significant (Fig. 2d). These findings suggest that vitamin C promotes ECM gene expression in a dose-dependent manner, supporting its potential role in anti-aging skin treatments.

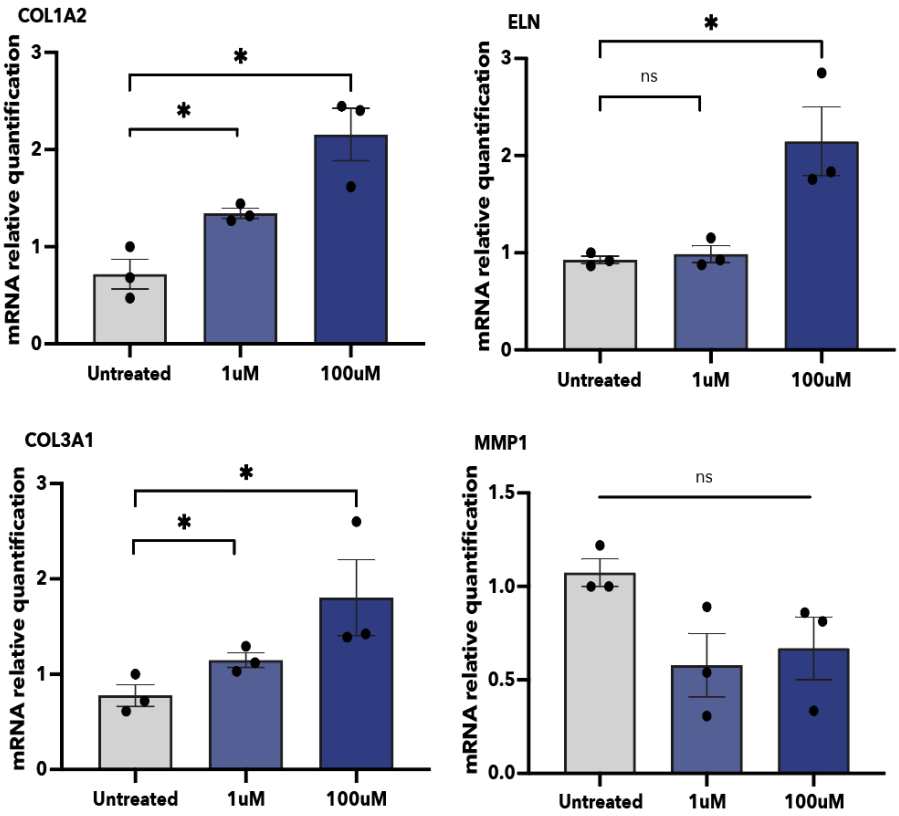
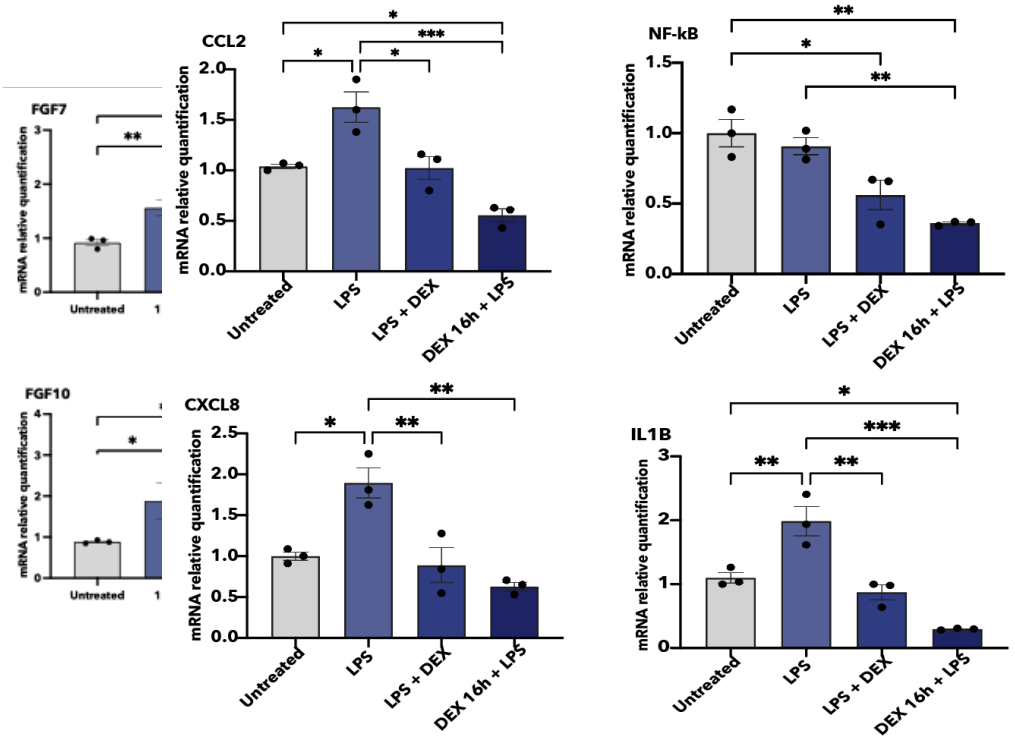



*Figure 2. Vitamin C Induces Dose-Dependent Upregulation of Extracellular Matrix Genes in Skin Organoids. (a–d) Quantitative PCR analysis of COL1A2, ELN, COL3A1, and MMP1 gene expression in mature skin organoids treated for 48 hours with 1 µM or 100 µM ascorbic acid. Expression levels were normalized to untreated controls. Data represent mean ± SD from triplicate biological replicates. Statistical analysis was performed using one-way ANOVA ($p < 0.05$).*

### 3.3 Modulation of LPS-Induced inflammatory response by dexamethasone in skin organoids

To establish an inducible inflammatory model in our skin organoids and evaluate their responsiveness to anti-inflammatory treatment, organoids were treated with 10 µM lipopolysaccharide (LPS) alone or in combination with 10 µM dexamethasone. An additional condition was included where organoids were pre-treated with dexamethasone for 16 hours prior to LPS exposure for 24 hours. Total RNA was extracted, and expression of pro-inflammatory markers *CCL2*, *NF-κB*, *CXCL8*, and *IL1B* was assessed by quantitative PCR (Fig. 3). LPS treatment significantly upregulated the expression of *CCL2*, *CXCL8*, and *IL1B*, while *NF-κB* levels remained unchanged. Co-treatment with dexamethasone attenuated the LPS-induced expression of these cytokines, bringing levels close to those of untreated controls. Notably, pre-treatment with dexamethasone led to an even greater reduction in inflammatory gene expression, suggesting enhanced anti-inflammatory efficacy when administered prior to inflammatory stimulation. These results demonstrate the potential of our organoid system for modeling skin inflammation and testing anti-inflammatory compounds.




*ces Expression Genes in Skin pression levels , IGF1, FGF10, ids treated with or 14 days. ata triplicate biolo- analysis was ANOVA (p <*

*Figure 3 Dexamethasone Suppresses LPS-Induced Inflammatory Gene Expression in Skin Organoids (a–d) Gene expression levels of CCL2, NF-κB, CXCL8, and IL1B in skin organoids treated with 10 µM lipopolysaccharide (LPS) for 24 hours, with or without 10 µM dexamethasone (Dex). Additional condition included pre-treatment with Dex for 16 hours followed by 24-hour LPS exposure. Data represent mean ± SD from triplicate biological replicates. Statistical analysis was performed using one-way ANOVA (p < 0.05).*

### 3.4 Minoxidil promotes hair growth–associated gene expression and follicular activity in skin organoids

To assess the potential of our skin organoids to respond to hair growth stimulation, we evaluated their response to minoxidil, a well-established reference compound for hair regrowth. Mature organoids were treated with either 1 µM or 10 µM minoxidil for 14 days, with media renewed every two days and fresh compound added at each change. At the end of the treatment period, total RNA was extracted and analyzed by qPCR for the expression of hair growth–related genes: *FGF7, IGF1, FGF10*, and *VEGFA* (Fig. 4). All four genes exhibited a

significant upregulation at both concentrations, with a modestly greater effect observed at 10 µM.

To validate these molecular findings, whole organoids treated with 10 µM minoxidil for 14 days were subjected to immunostaining. Keratin 17 staining was used to localize the outer root sheath of hair follicles, while Ki67 identified proliferative cells within the follicular regions (Fig. 5a). Quantification revealed an increase in hair follicle volume and a marked augmentation in the number of Ki67-positive cells per follicle in the minoxidil-treated group compared to vehicle controls (Fig. 5b-c). These results confirm that skin organoids are responsive to minoxidil and demonstrate structural and molecular changes consistent with enhanced hair follicle activation.

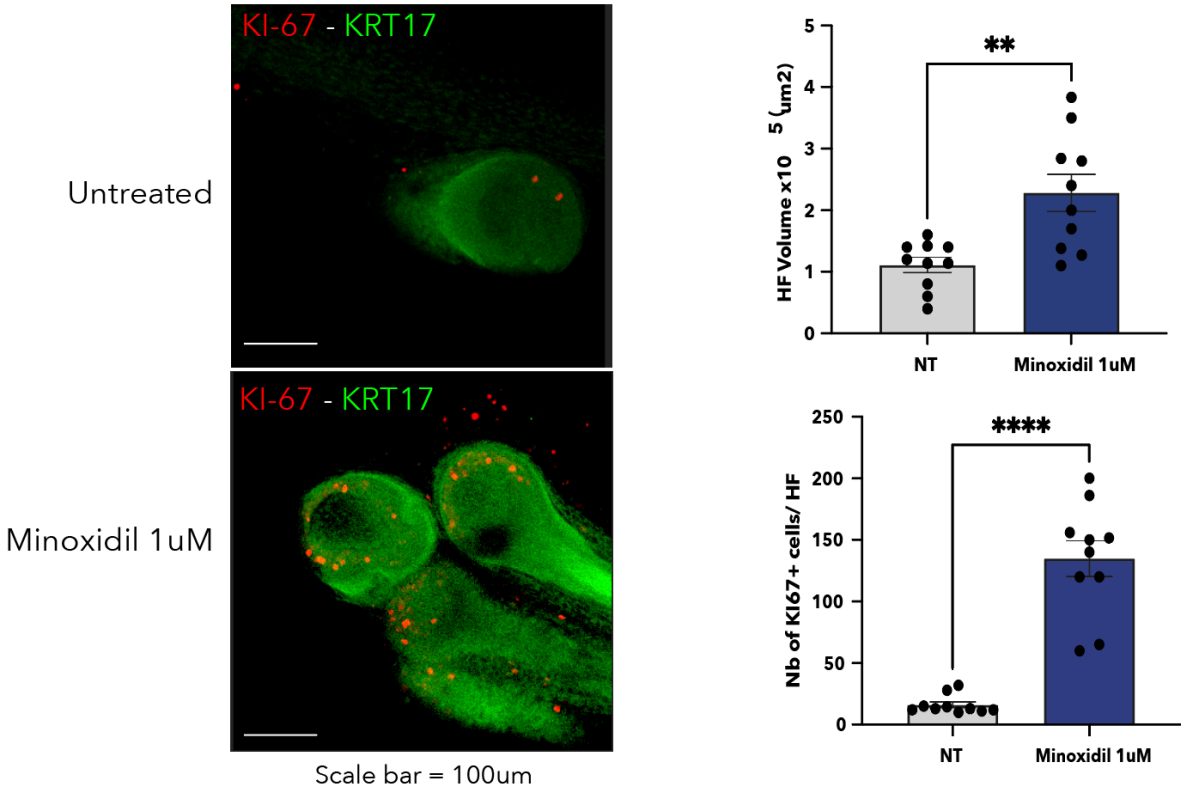



*Figure 5. Minoxidil Promotes Hair Follicle Enlargement and Proliferation in Skin Organoids (a) Immunostaining of whole-mount organoids treated with 10 µM minoxidil for 14 days. Keratin 17 (green) marks the outer root sheath of hair follicles; Ki67 (red) identifies proliferative cells within the follicular structure. Nuclei counterstained with DAPI (blue). (b) Quantification of hair follicle volume and Ki67-positive cells per follicle shows a significant increase in Minoxidil-treated organoids compared to vehicle control. Data represent mean ± SEM from multiple follicles per condition ($p < 0.05$).*

*Link to reconstructed videos*
*NT: https://scanned.page/p/678e4fe7307e1*
*Minoxidil : https://scanned.page/p/678e020b3221a*

## 4. Discussion

Recent advances in three-dimensional (3D) *in vitro* modeling have transformed toxicity testing by offering physiologically relevant alternatives to traditional animal models. Historically, animal testing was considered the gold standard due to the lack of systems that could replicate whole-organism complexity. However, with the rise of ethical concerns and the limited translatability of animal data to human biology, particularly in cosmetics, in vitro assays have become the norm. In this context, our customizable skin organoid model demonstrates significant potential as a robust platform for cosmetic safety and efficacy

assessments. When tested with reference molecules listed under OECD Guideline 439 [7], our system displayed consistent and reproducible viability outcomes, outperforming conventional Reconstructed Human Epidermis (RHE) models in terms of inter-assay variability. One current limitation is the spherical architecture of the model, which lacks an air–liquid interface and therefore does not develop a stratified stratum corneum. As a result, compound testing is limited to systemic exposure via the culture medium, restricting its suitability for irritation studies.

Given its inclusion of both dermal and epidermal compartments, we assessed our model's responsiveness to ascorbic acid, a compound known to stimulate collagen production and act as an antioxidant in fibroblasts and skin explants [23], [24]. The treatment upregulated extracellular matrix gene expression, in line with literature, and upcoming work will include whole-organoid collagen fiber staining to confirm these effects.

We also explored the inflammatory potential of the model using lipopolysaccharide (LPS) stim-ulation, which successfully induced pro-inflammatory gene expression. Co-treatment with dexamethasone effectively suppressed these responses, suggesting the model's utility for evaluating anti-inflammatory properties of dermocosmetic candidates. To further validate this capability, a characterization of secreted cytokine profiling will be undertaken.

A unique feature of our organoid is the presence of skin appendages, notably hair follicles, which are rare in standard skin models. Thus, we evaluated the response to minoxidil, a well-established treatment for alopecia. Although minoxidil's mechanism of action remains partially unresolved, it is generally thought to prolong the anagen phase of hair growth by enhancing blood flow and nutrients supply via peripheral vasodilation [25]. While our organoids lack a vascular component, prior studies have identified expression of the sulphonylurea receptor (SUR2B) —minoxidil's presumed target present in vascular smooth muscles cells—in human dermal papilla cells [26]. Based on this, we hypothesized that minoxidil may exert direct effects on follicular cells independent of vascular signaling. Accordingly, minoxidil treatment led to upregulation of key hair growth genes and increased follicle size and proliferation. These findings support the application of our skin organoids as a comprehensive in vitro platform to study not only toxicity but also regenerative and therapeutic properties of dermatological compounds

## 5. Conclusion

In conclusion, our skin organoid model represents a versatile and physiologically relevant *in vitro* platform for cosmetic and dermocosmetic testing, capable of assessing cytotoxicity, inflammation, and hair growth modulation with high reproducibility and functional complexity.

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