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"Advanced skin organoid that perfectly mirrors real skin ~The evolution of an innovative skin model to develop the ultimate personalized cosmetics~"

Moena Takahashi [1,*], Yuki Shirai [1], Tomoki Nishiguchi [1], Aoi Mori [1], Mizuho Nakano [1] and Tomoyuki Iwanaga [1]

[1] POLA CHEMICAL INDUSTRIES, INC., FRONTIER RESEARCH CENTER, Yokohama, Japan

1. Introduction

In recent years, there has been increasing interest in developing personalized cosmetics to meet diverse beauty standards. While various skin models are employed to evaluate cosmetic ingredients [1], a novel skin model called "Mirror Skin 1.0", which is derived from induced pluripotent stem cells (iPSCs) generated from donor urine cells, has been recently developed [2–5]. Mirror Skin 1.0 exhibits UV responsiveness that matches that of the donor skin, while retaining donor-specific features. However, its spherical structure with the dermis placed over the epidermis, does not reflect the correct configuration of human skin. To address this issue, we developed "Mirror Skin 2.0", a flat model with the epidermis placed correctly over the dermis as in actual human skin. Mirror Skin 2.0, which expresses tight junction (TJ) proteins and demonstrates barrier functions similar to those of human skin, is suitable for comprehensively evaluating cosmetic ingredients. As an iPSC-derived model, Mirror Skin 2.0 is estimated to retain donor-specific characteristics and provides an ideal platform for developing personalized cosmetics.

2. Materials and Methods

Generation of Mirror Skin 2.0 from iPSCs

iPSCs (Takara Bio) were cultured in vitronectin-coated 6-well plates in Essential 8 Flex medium (Thermo Fisher Scientific; hereafter, E8). At approximately 80% confluency, cells were detached using TrypLE Select (Thermo Fisher Scientific) and seeded at 3,500 cells/well into U-bottom 96-well plates with E8 medium containing 20 μM Y-27632 (FUJIFILM Wako). Aggregates were formed by centrifugation at 100 ×g and induced to differentiate into spheroids as previously described [6,7]. On Day 4 after the induction of differentiation, the spheroids were transferred onto cell culture inserts. The cell culture inserts used were either FibColl Atelocollagen Inserts 24 (Koken, as is), or Transwell cell culture inserts (Corning, coated with

type I collagen). The spheroids were maintained for over 100 days to mature into Mirror Skin 2.0. Culture medium was added only to the bottom well of the insert, and its composition was based on previously reported protocols [6,7].

H&E staining and immunofluorescence staining

On approximately Day 100, Mirror Skin 2.0 was harvested and processed for both paraffin and frozen embedding. Paraffin sections were stained with hematoxylin and eosin (H&E) (SKK Tissue Science Research Laboratory) and imaged using a NanoZoomer S60 (Hamamatsu Photonics). Frozen sections were used for immunohistochemical analysis using the antibodies listed in Table 1 and mounted using ProLong Gold with DAPI (Thermo Fisher Scientific). Fluorescence images were captured using a BZ-X810 microscope (Keyence).

Morphological evaluation of the spheroids

The spheroid morphology was evaluated to determine the optimal timing for transferring them onto the insert in the Mirror Skin 2.0 fabrication process. The spheroids cultured using conventional methods [6,7] collected from Day 3 to 18 were sectioned and stained with E-cadherin and PDGFRα antibodies.

Functional assay of TJ

TJ formation was investigated based on the impermeability of Sulfo-NHS-LC-Biotin [8,9]. Mirror Skin 2.0 was excised from the insert and transferred to a dish. 2 mg/mL Sulfo-NHS-LC-Biotin was applied dropwise onto the epidermal side and incubated for 10 min. The sample was then embedded in O.C.T. compound (Sakura Finetek) and sectioned at 10 μm. Sections were co-stained with ZO-1, and incubated with a secondary antibody and 10 μg/mL Streptavidin-Alexa Fluor 647 conjugate. Images were captured using a BZ-X810 microscope (Keyence).

Table 1. Antibody information

Name	Stained region	Supplier	Dilution
E-cadherin	Epidermis	BD Biosciences	1:500
PDGFRα	Dermis	Cell Signaling	1:500
SOX2	Dermal papilla	Invitrogen	1:100
KRT5	Basal keratinocyte	Cell Signaling	1:100
PMEL	Melanocyte	GeneTex	1:100
KRT10	Intermediate keratinocyte	Abcam	1:100
ZO-1	Tight junction	Proteintech	1:100
Sulfo-NHS-LC-Biotin	-	Thermo Fisher Scientific	-
Streptavidin-Alexa Fluor 647 conjugate	-	Thermo Fisher Scientific	-
Alexa Fluor 488	-	Abcam	1:1000
Alexa Fluor 555	-	Invitrogen	1:1000
Alexa Fluor 568	-	Abcam	1:500
Alexa Fluor 647	-	Abcam	1:1000

3. Results

Generation of Mirror Skin 2.0 with a flat-surfaced structure

The spheroids were cultured on cell culture inserts under an air-liquid interface (ALI), with the basal side in contact with the medium and the apical side exposed to air (Figure 1a). By Day 50, the cells had spread across the insert, and hair follicle-like structures appeared around Day 80 (Figure 1a). On approximately Day 100, a flat-surfaced skin organoid, Mirror Skin 2.0, was formed, with the epidermis positioned at the top and the dermis below. H&E staining confirmed the presence of the epidermis, dermis, adipose tissue, and hair follicles (Figure 1b–d).

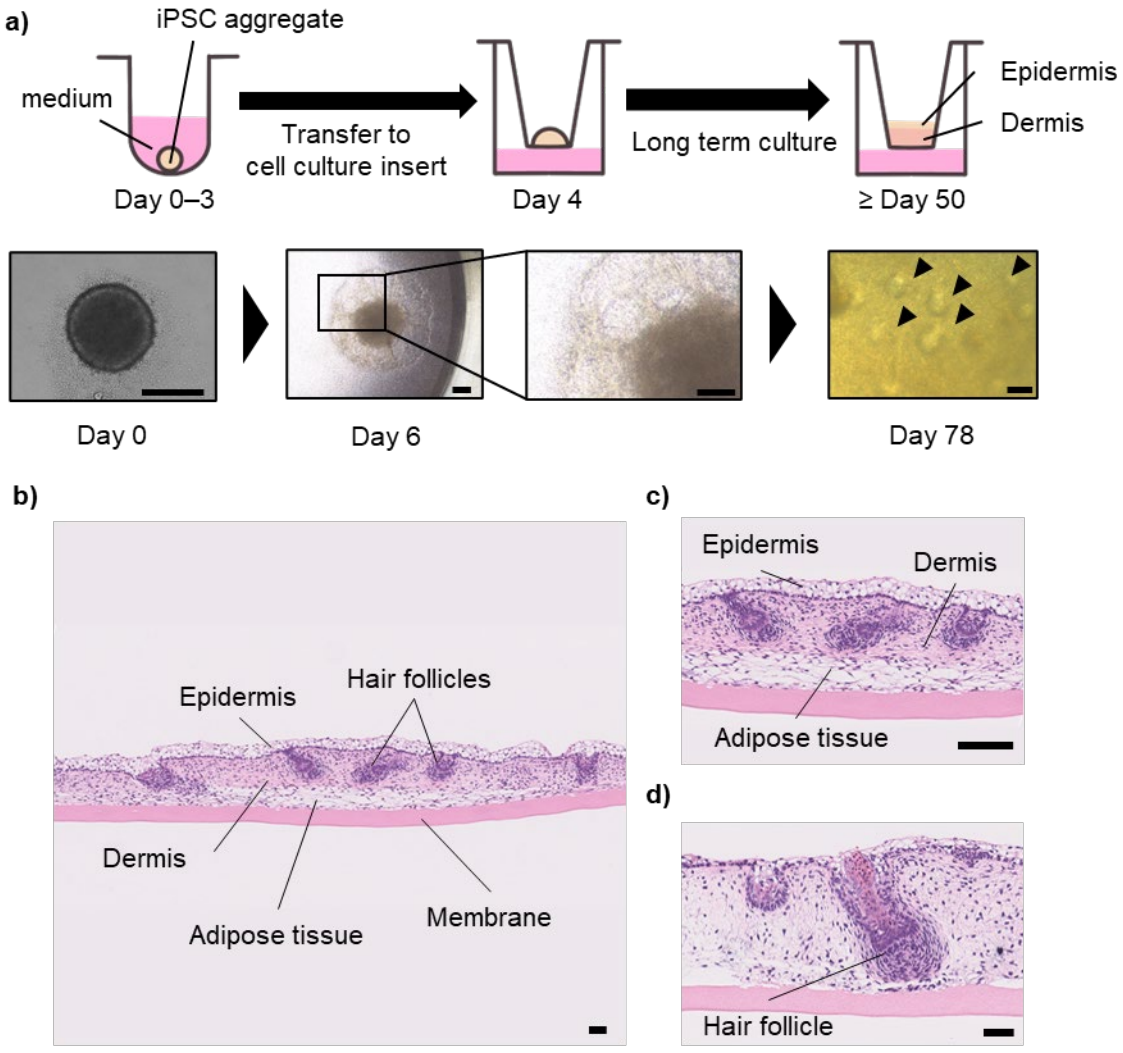

Figure 1. Characterization of Mirror Skin 2.0 with a flat-surfaced structure

a) Method of generating Mirror Skin 2.0 and external view of the organoid. Arrow heads indicate the location of hair follicles. b–d) H&E staining images. Epidermis, dermis, adipose tissue, and hair follicles. b) Low magnification image of Mirror Skin 2.0. c) Enlarged image of b. d) Another section of the same specimen shown in b. Scale bars: a, 150 μm; b–d, 50 μm.

Spheroids capable of maturing into Mirror Skin 2.0

When spheroids cultured from Day 6 to 18 were transferred to the insert, some changed to a flat-surfaced structure from the spherical structure, while others retained their spherical structure. However, these conditions presented challenges such as the failure of hair follicle maturation. This tendency became more pronounced with longer culture durations, as indicated by an increase in internal cavitation within the spheroids. These observations suggest that, similar to Mirror Skin 1.0, the spheroids tend to mature into a spherical configuration (Figure 2). Therefore, we examined spheroids at earlier culture stages and found that those on Day 4 exhibited the most favorable conditions for the successful maturation of Mirror Skin 2.0.

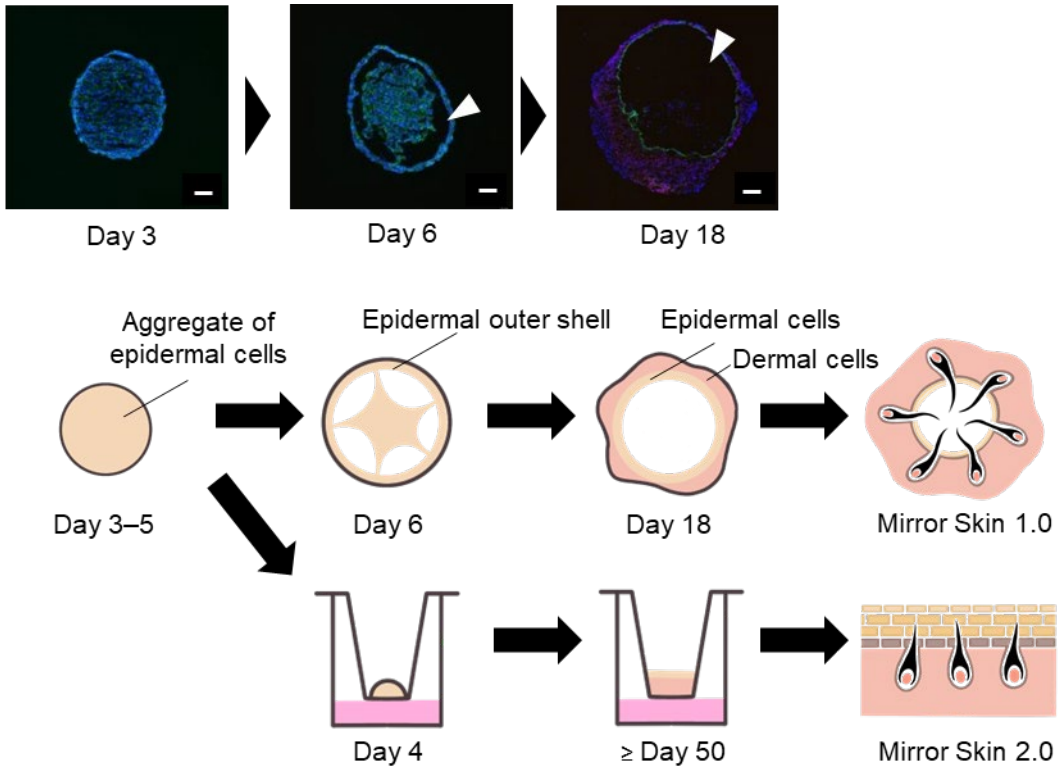

Figure 2. Morphological changes of the spheroids

Schematic diagram showing the morphological changes in spheroids and their morphology from Day 3 to 18. The white arrow heads indicate the internal cavitations. Scale bars: 100 μm.

Structure of Mirror Skin 2.0

Immunohistochemical analysis revealed that on Day 116, Mirror Skin 2.0 exhibited an organized structure with a PDGFRα$^+$ dermis beneath an E-cadherin$^+$ epidermis (Figure 3a). SOX2$^+$ dermal papilla cells, KRT5$^+$ basal keratinocytes, and PMEL$^+$ melanocytes were also identified (Figure 3a, b). Furthermore, the expression of ZO-1 confirmed the formation of TJs, which are essential for epidermal barrier function (Figure 3c, d).

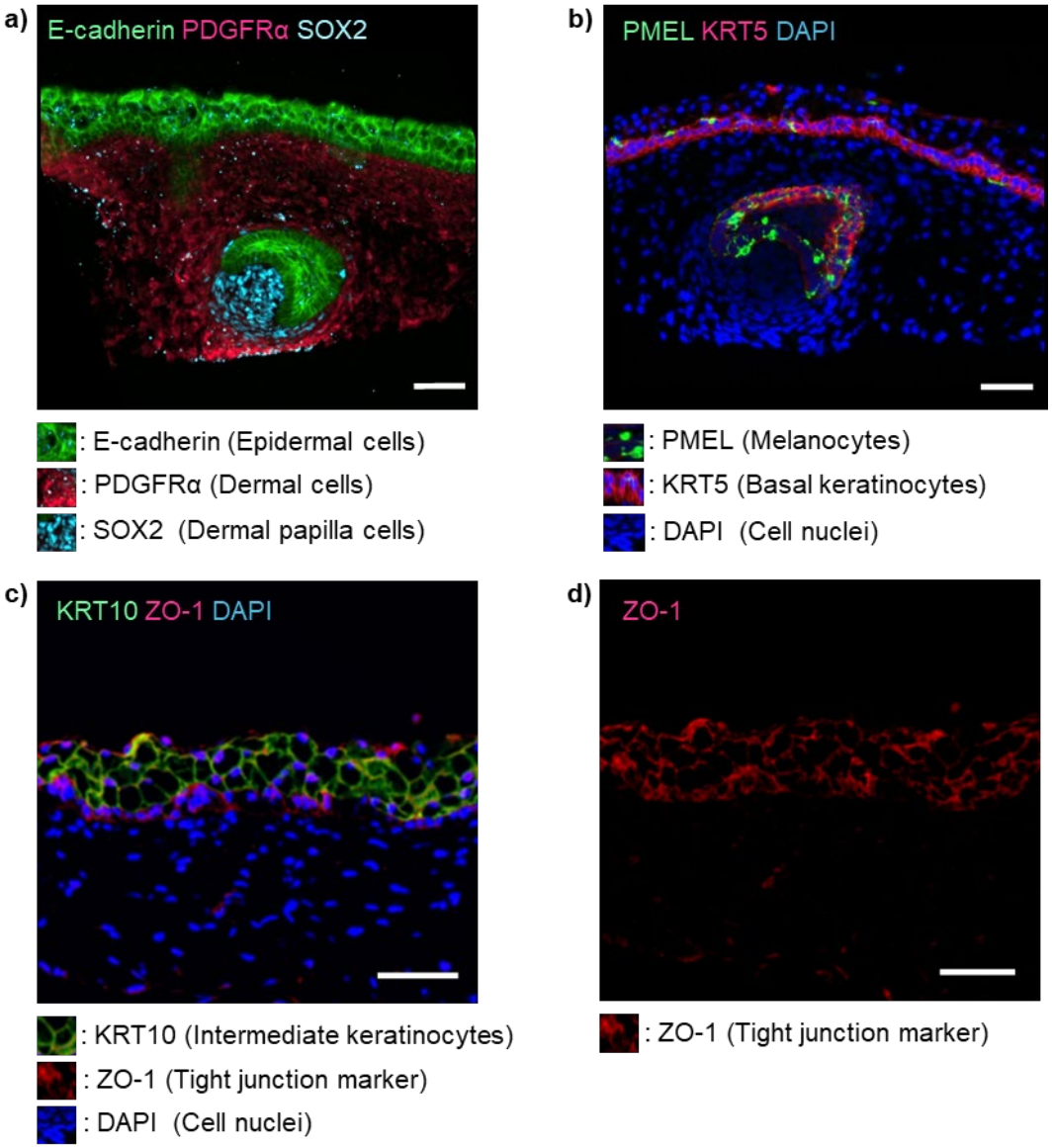

Figure 3. Protein expression in Mirror Skin 2.0

a–d) Immunofluorescence images of Day 116 Mirror Skin 2.0. a) The E-cadherin$^+$ epidermis is indicated in green, the PDGFRα$^+$ dermis is indicated in red, and SOX2$^+$ dermal papilla cells are indicated in light blue, showing the skin structure and a hair follicle. b) PMEL$^+$ cells in the KRT5$^+$ epidermal basal layer indicate the presence of melanocytes. c) The TJ marker ZO-1 was expressed in the KRT10$^+$ epidermal intermediate layer. d) A fluorescence image displaying only the ZO-1 signal. Scale bars: 50 μm.

Functional evaluation of TJs in Mirror Skin 2.0

Next, a permeability assay was conducted to confirm whether TJs, that are present in the epidermis of Mirror Skin 2.0, possess epidermal barrier functions. Sulfo-NHS-LC-Biotin (MW 556.59), whose skin permeation would be inhibited by TJs if functional, was dropped onto the epidermis of Mirror Skin 2.0. The same sample was co-stained with ZO-1 antibodies (Figure 4a). ZO-1 and Sulfo-NHS-LC-Biotin are highlighted in Figure 4b and Figure 4c as red and green, respectively. Figure 4d shows the coexistence of ZO-1 and Sulfo-NHS-LC-Biotin (indicated in yellow) that does not permeate through the lower border of TJ, indicating that Mirror Skin 2.0 has functional TJs (Figure 4d).

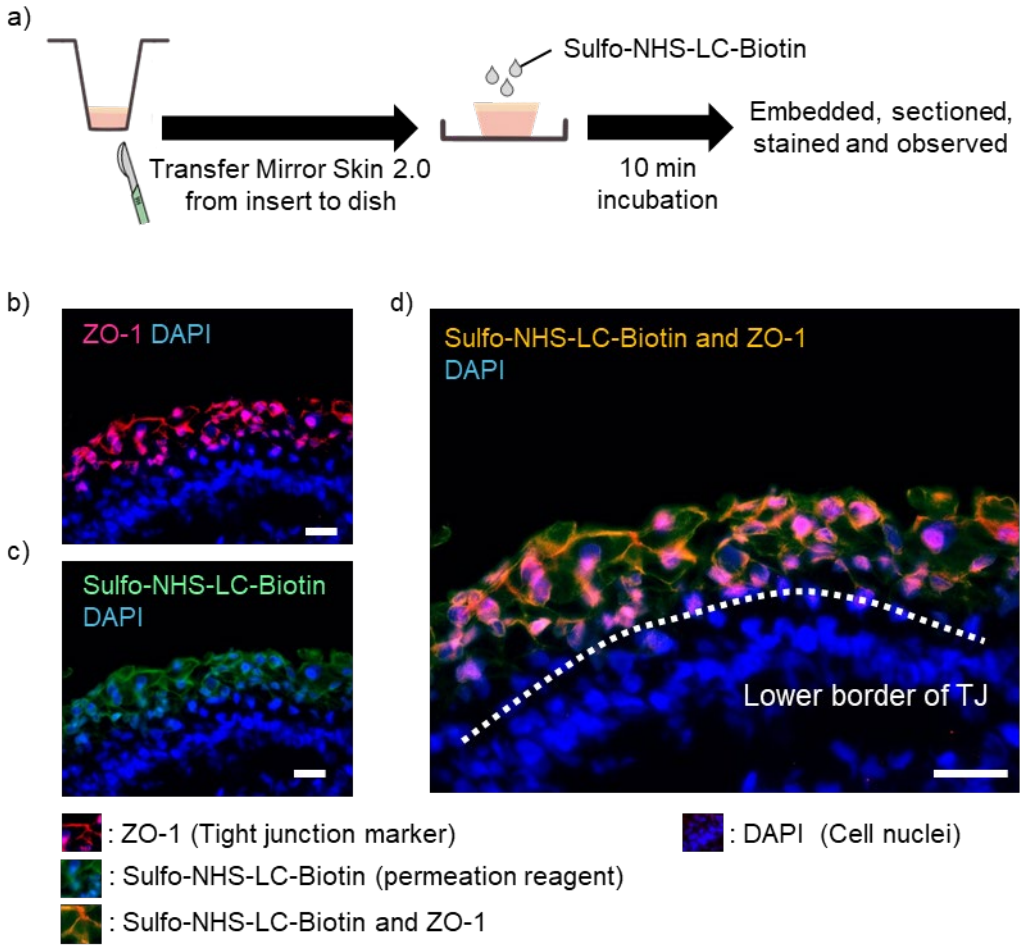

Figure 4. Functional evaluation of TJs in Mirror Skin 2.0

a) Method of permeability assay. b–d) Fluorescence images of permeability assay. Green indicates the regions where biotin has permeated, red represents the expression of ZO-1, and blue marks the cell nuclei. b) Fluorescence image of ZO-1. c) Fluorescence image of the Sulfo-NHS-LC-Biotin. d) Fluorescence image of the co-staining with Sulfo-NHS-LC-Biotin and ZO-1 antibody. Scale bars: 20 μm.

4. Discussion

Mirror Skin 2.0 maintained the complex cell population observed in Mirror Skin 1.0 and contained key skin components, such as melanocytes, hair follicle-associated cells like dermal papilla cells and adipocytes, highlighting its advantage over conventional artificial skin bilayer models. Mirror Skin 2.0 features a flat-surfaced structure, and functional evaluation of TJs suggests the presence of a barrier function. Therefore, it is a suitable skin model for evaluating cosmetic ingredients that require epidermal application, as well as for permeability assays.

The development of this model required the culture conditions to be optimized. In this study, spheroids were cultured under ALI conditions. For proper cellular differentiation, it is essential to provide an air culture environment that closely resembles the native biological environment in which the cells reside [10]. We hypothesized that the use of ALI culture could replicate the native environment of human skin, wherein the epidermis is exposed to air and the dermis has vasculature and is nutrient rich.

However, under certain conditions, the spheroids failed to form a flat-surfaced structure. In the initial experiment, spheroids from Day 6 to 18 were used. When spheroids collected before Day 12, during the initial emergence of dermal cells, were cultured under ALI conditions, a flat-surfaced structure was observed in some cases, but no mature hair follicles were detected. Notably, Day 6 spheroids appeared to have already initiated the formation of an epidermal outer shell, which may have restricted epithelial cell motility by establishing apical-basal polarity in the epidermal basal layer. This restriction likely hindered structural maturation.

To address these issues, we focused on using Day 4 spheroids, which are considered to retain sufficient cell motility. This adjustment enabled the successful generation of Mirror Skin 2.0, which featured more mature hair follicles and a structured epidermis positioned above the dermis. These results suggest that the use of Day 4 spheroids, which retain undifferentiated and motile cellular characteristics, in combination with a culture environment that mimics the biological properties of native skin, is critical for the development of Mirror Skin 2.0.

Moreover, due to its structural characteristics and cellular composition closely resembling those of human skin, Mirror Skin 2.0 represents a promising model for various skin-related applications. These include cosmetic evaluation, safety and efficacy testing, and research on skin problems such as pigmentation and wrinkle formation.

5. Conclusion

We successfully established an improved model that surpasses Mirror Skin 1.0 in both structure and function. Mirror Skin 2.0, developed in this study was constructed to possess a flat surface, reflecting actual human skin. Moreover, the expression of proteins involved in TJs, essential for barrier function, was observed, and their functionality was validated through the TJ assay. Taken together, Mirror Skin 2.0 serves as a skin model suitable for evaluating active cosmetic ingredients, with consideration of skin barrier function.

Mirror Skin 2.0 is expected to be an optimal skin model for the development of ultimate personalized cosmetics.

In this study, we conducted a permeability assay using Mirror Skin 2.0; however, it is important to note that the stratum corneum also plays a critical role in the skin's barrier function,

and we are currently working on how to create a mature stratum corneum to further enhance this skin model. In the future, we aim to further advance Mirror Skin 2.0 and develop an optimized skin model that can serve as a platform for evaluating cosmetics, ultimately contributing to the creation of ultimate personalized cosmetics.

6. References

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