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# Bioprinting technology to build a new equivalent skin model with sebaceous gland-like structures



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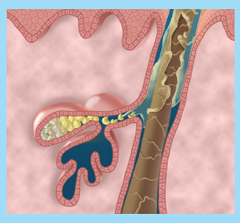
## INTRODUCTION

Transition from *in vitro* to clinical trials presents a huge gap that has mainly been filled with animal testing. However, testing on animals are not always reliable because they remain physiologically different from human and are banned in the cosmetic industry, notably in Europe. Therefore, the need for reliable in vitro models is increasing to test molecules [1, 2]. 3D models for different organs have been set up [3]. These models are physiologically more coherent than 2D models, and present advantages in data reliability and are more predictable for clinical trials.

Sebaceous glands are holocrine glands. Most sebaceous glands are associated to hair follicles, but in some locations, they arise independently of the hair (e.g. Meibomian glands in eyelids). Sebaceous glands secrete sebum on the skin surface, therefore contributing to 90 % of skin lipids. Sebum plays a role in hydration, thermoregulation, and microbial protection [4, 5, 6].

3D models with sebaceous glands consists in: skin explants, sebaceous gland explants or 3D skin organoids [6]. Skin explants are collected during plastic surgery. These models are difficult to procure especially during COVID19 pandemics. Skin explants are hard to cultivate, and we only obtain a limited number of samples. Furthermore, donor-donor heterogeneity is high, and explants cannot always be reliable to model a general response. Sebaceous glands can also be cultured as isolated organs [7]. However, these models remain difficult to cultivate and present donor-dependent heterogeneity. Nowadays, 3D sebaceous glands models are generated by single culture of immortalised human sebocytes in extracellular matrices. Each cell divides and differentiates in a sebaceous gland- like structure [8, 9]. Despite using immortalised cell, this last model does not mimic skin environment either. There is therefore a need to generate bioengineered 3D skin models containing sebaceous gland- like structures.



Bioprinting has become an essential tool in skin bioengineering. Bioprinting technologies can be grouped in nozzle-based and nozzle-free technologies. All technologies have pros and cons such as cost, reliability, speed, resolution, cell viability and handling of printed skin samples due to their size.

To minimize drawbacks, we decided to use multimodal bioprinter to develop a 3D skin model containing structures similar to sebaceous glands [10].

In this poster we describe the process from setting-up of sebocyte printing parameters to the generation of a full-skin model containing sebaceous gland like structures.

## MATERIAL AND METHOD

### Bioprinting

Two different technologies were combined to print in 3D the equivalent dermis. A microvalve technology was used to print the collagen layers whereas laser-assisted bioprinting was used for the cell layers.

**Dermis bioprinting and maturation:**

Prior printing, fibroblasts were cultured in DMEM/F12 media containing antibiotics and 10 % serum. Rat collagen type I was used with dermal human fibroblasts. The 3D structure was created by alternating layers of collagen and layers of fibroblasts. Maturation duration was defined to obtain the best timing before keratinocytes printing (1 to 5 days). For dermis maturation we used DMEM/F12 with 10 % FCII, 5-50 µg/mL of ascorbic acid and contained antibiotics.

**Epidermis bioprinting and proliferation:**

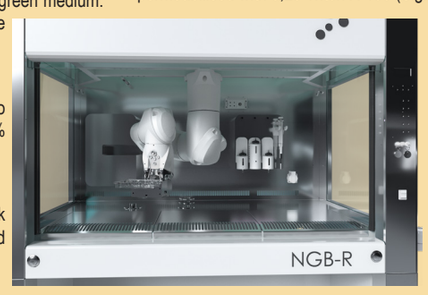
Prior printing, keratinocytes were cultured in CnT-Prime keratinocyte medium containing antibiotics. The epidermis basal layer was designed with a pattern that ensures a uniform distribution of epidermal human keratinocytes. After bioprinting, epidermal proliferation was performed in submerged conditions in green medium. Proliferation duration was defined to obtain the best timing before differentiation.

**Epidermal differentiation:**

Differentiation duration at the air liquid interface (ALI) was defined to obtain *stratum corneum* layers. Modified green medium with 0.8 % BSA was used to enhance epidermal differentiation.

**Sebocyte bioprinting:**

Sebocyte were bioprinted during dermal production. A bioink containing sebocytes was prepared. Spots of sebocytes were printed as described in the result section




### hiPSC-SEB cell culture

Human sebocytes obtained with the reprogramming of induced pluripotent stem cells technology (iPS) were provided by Phenocell (France). Cells were routinely cultured with 5 % $CO_2$ at 37°C and 90 % humidity atmosphere in regular Phenocult-SEB medium (Phenocell, France) according to manufacturer's instructions.

### Viability staining

Viability staining was performed using live/dead viability/cytotoxicity kit (Invitrogen, USA) according to manufacturer's instructions.

### Staining

Models were embedded in freezing media (Leica, Australia) and snap-frozen in liquid nitrogen. Sections were prepared using cryostat (CM15105, Leica, Germany). Histology was evaluated by Masson-Goldner's trichrome staining. For lipid (Bodipy, Invitrogen) and FASN (abcam, USA) staining, sections were fixed in 4 % paraformaldehyde (Sigma, USA), blocked in 2 % BSA (Sigma, USA) and permeabilised with 0,25 Triton X-100 (Sigma, USA).

### qPCR

RNA extraction was performed using RNeasy Fibrous Tissue Mini Kit (Qiagen, Germany). RNA quantity was assessed using Nanodrop and 1µg RNA was reverse transcribed using iScript cDNA Synthesis kit (Biorad, USA). Gene expression was assessed using SYBRGreen (Biorad, USA) and CFX technology (Biorad, USA).

## RESULTS

### 1- Sebocyte bioprinting on a collagen lattice

Human sebocytes were printed using parameters set up with rodent sebocytes. Similar to rodent cells (data not shown), human sebocytes were printed on a collagen lattice and aggregates of viable cells were visible at 24h post-bioprinting. Aggregates of viable cells were visible at 24h post-bioprinting. However, we observed that aggregates of hiPSC-Seb were more dispersed, they divided less and were lost already 48h after printing. In order to help aggregate maintenance, we increased number of sebocytes per spot and inter-spot spacing to reduce cell attraction between different spots (Fig 1A). Using these new settings, we managed to have isolated aggregates for at least 6 days.

We then decided to study how different media could affect our aggregates. Printed hiPSC-Seb were cultivated in their growth media, in dermal maturation media or in immersion media for 6 days. Only dermal maturation media allowed maintaining cells in small aggregates (Fig 1B). We therefore cultivated our models for 4 days in dermal maturation media followed by 3 days of immersion media. In these conditions aggregates were maintained but they seemed to detach from the collagen lattice layer which would cause loss of spheres during further experimentations. To solve this issue, we covered sebocytes with a layer of collagen. We could therefore keep our sebocytes for at least 11 days (Fig 1C).

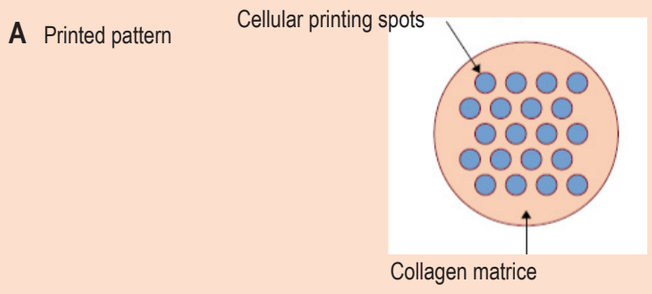



Figure 1: Biopinting of hiPSC-sebocyte on collagen lattice A: Bioprinting pattern.
B: Culture with sebocyte media, skin immersion media or dermal maturation media. Maintained aggregates when cultured in dermal maturation media.
C: Avoiding aggregate detachment by embedding in collagen gel.

### 2- Equivalent dermis with sebaceous gland-like structures

After having managed to produce viable aggregates that could be kept in culture for at least 11 days we generated equivalent-dermal models with sebaceous-like structures. To do so, spots of sebocytes were printed during dermal bioprinting process as showed in Fig 2A. Individual aggregates were maintained during dermal contraction (Fig 2B). Dermal models were cut, and morphology was studied. Sebaceous structures were visualised by Masson-Goldner trichrome staining and lipid staining (Fig 2B).

To study functionality of our aggregates we treated dermal models with a compound known to induce lipid production and evaluated lipid content as well as FASN (Fatty Acid Synthase) an enzyme involved in lipid production. We showed that pro-sebum treatment increased FASN quantity and lipid content (Fig 2C). We also evaluated gene expression of Plin2 coding for a protein located in the membrane of lipid droplets. Plin2 gene expression was increased 2-fold compared to vehicle ($p<0.05$) 48h after treatment.

We therefore proved the functionality of our dermal model containing sebaceous gland-like structures.

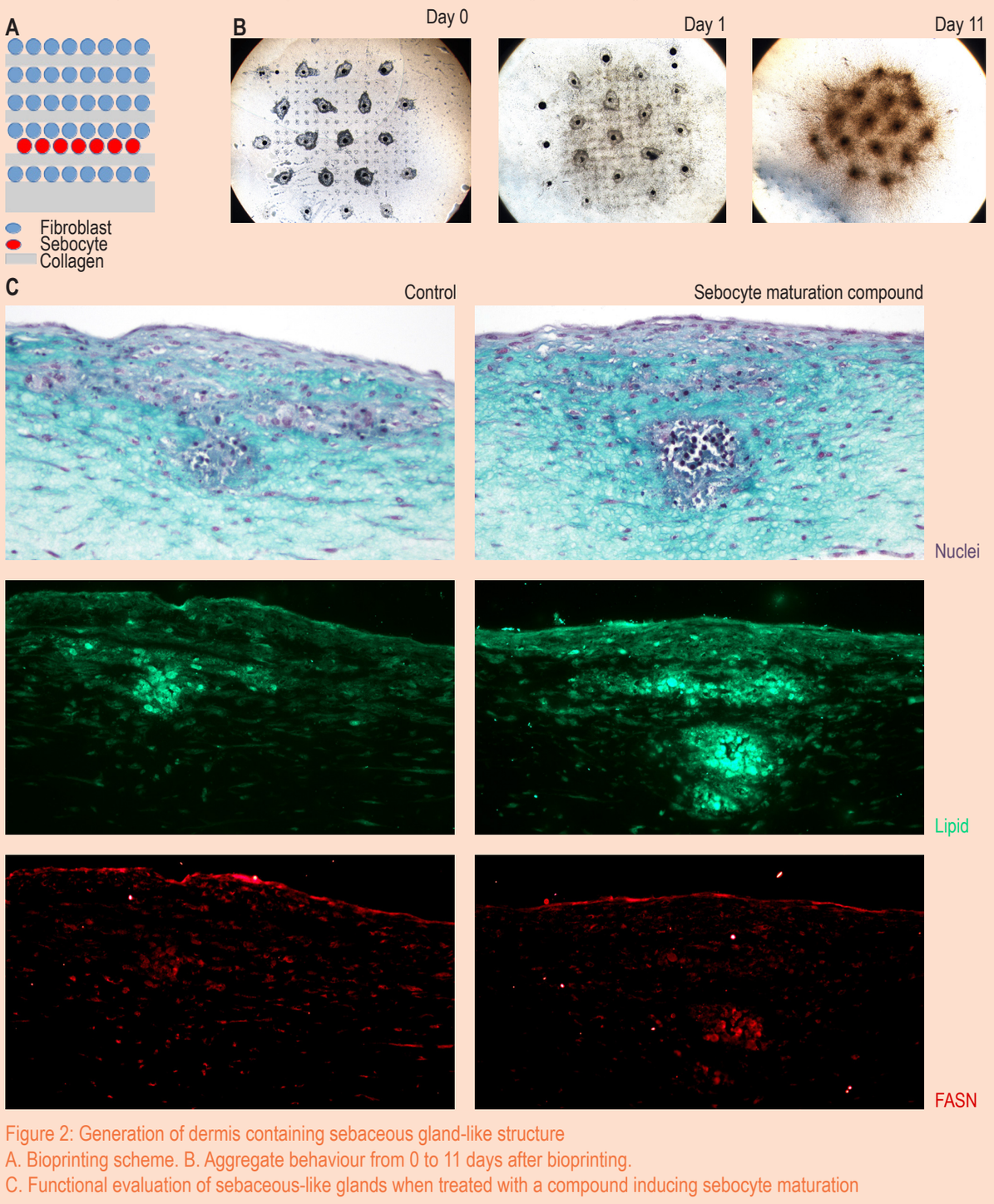



Figure 2: Generation of dermis containing sebaceous gland-like structure
A. Bioprinting scheme. B. Aggregate behaviour from 0 to 11 days after bioprinting.
C. Functional evaluation of sebaceous-like glands when treated with a compound inducing sebocyte maturation

### 3- Full skin with sebaceous gland-like structures

After having successfully embedded sebaceous gland-like structures into equivalent dermal models, we bioprinted full skin models with sebocytes as shown in Figure 3A. Surprisingly, aggregates migrated toward epidermis. We stained our models with Masson-Goldner trichrome for histology evaluation (Fig 3B). Models were stained for lipids and visualised by light sheet microscopy (Fig 3C). Epidermal structure was however not optimal. Further studies are necessary and will focus on improvement of our model.

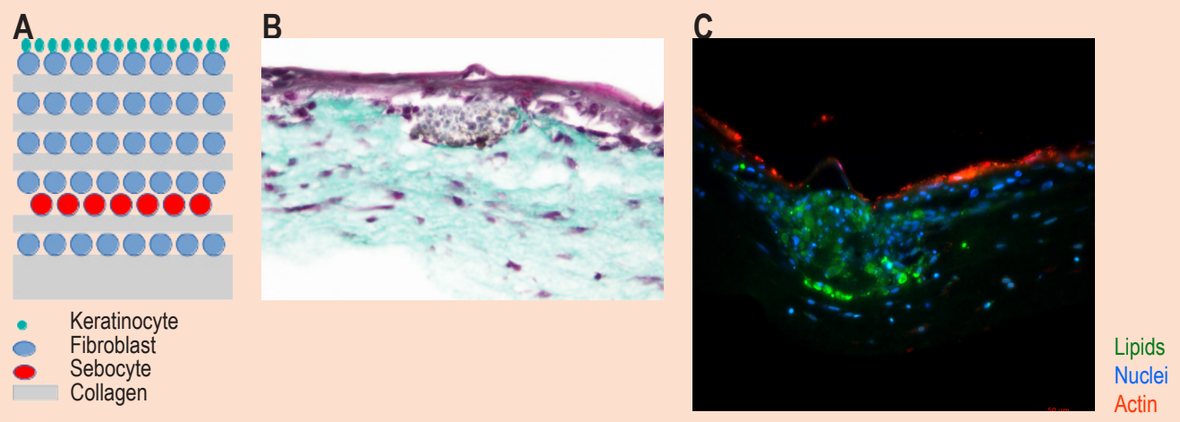



Figure 3: Generation of full-skin containing sebaceous gland-like structure
A. Bioprinting scheme
B. Skin histology, H&E staining
C. Lipid staining visualised with light sheet microscopy

## CONCLUSION

In this study we produced dermal and full-skin models containing hiPSCs sebocytes. A sequential Go/No-Go process was followed. We first set-up bioprinting parameters using a low-cost and easy to handle sebaceous cell line. We then adapted bioprinting parameters to hiPSCs sebocytes. Using these new established parameters, we produced equivalent dermal models with sebocytes. After having demonstrated that these structures responded to compounds, we produced full skin models.

One of the difficulties to manage, when working with our models, is the evaluation of compound's effect. Skin sectioning is one alternative. However, interpretation of the effect depends on the cutting location. Indeed, precisely finding a section including the sebaceous gland is somewhat complicated. Light sheet microscopy would be a more suitable technology, but further studies need to be performed to improve this.

Regarding full skin models, we observed that epidermis stratification was not optimal. This can be explained by different hypothesis. When printed deep in the dermis, we observed that sebaceous structures migrated near the epidermis. This could explain a mechanical disruption of epidermal layer. Another possibility is that dermal matrix is not rich enough to support epidermal stratification. Future experiments will focus on improving epidermal structure.

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