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

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Key words: *Skin models, bioprinting, human sebocytes*

Abstract

Background:

Sebaceous glands are holocrine glands that secrete sebum on the skin surface. Sebum plays a role in hydration, thermoregulation, and microbial protection. Currently 3D models with sebaceous glands consist of skin explants, sebaceous gland explants or 3D skin organoids. Skin explants and isolated sebaceous glands are difficult to procure, hard to cultivate, provide only a limited number of samples, and present donor-donor heterogeneity. There is therefore a need to generate bio-engineered 3D skin models containing sebaceous gland-like structures.

Method used:

Bioprinting has become an essential tool in skin bioengineering. Bioprinting technologies can be grouped in nozzle-based and nozzle-free technologies. For this study we used an hybrid bioprinter combining both technologies.

We first set up sebocyte bioprinting parameters using an affordable and easy to cultivate rodent sebaceous cell line, before switching to a human sebocytes derived from hiPSCs (human induced Pluripotent Stem Cells).

Results

After generating spheroids by printing spots of rodent sebocytes on a collagen layer, we switched to hiPSC-derived sebocytes which generated viable aggregates of 150µm of

diameter. When treated with compounds inducing terminal differentiation, markers such as FASN (Fatty Acid Synthase), PLIN2 (Perilipin-2) and accumulated lipid droplets were increased. Finally, sebaceous structures were included into full skin models.

Conclusion

Our model adds to the 2 existing bio-engineered skin models containing hiPSC-derived sebocytes. We are hoping to use it in evaluation of compounds acting in multicellular level.

Further studies will focus on improving our full-skin model and testing inhibitors and other inductors of sebocyte maturation.

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.

Skin models have first been generated as a graft alternative for patients with severe wounds and burns [4]. These full-skin models have been developed to reproduce a skin equivalent containing human primary fibroblasts embedded in a collagen matrix and human primary keratinocytes that stratify into an epidermis [4,5]. These models are used *in vitro* in both medical and cosmetic research. However, they lack skin appendages such as sebaceous glands.

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 [6,7, 8].

Sebaceous glands are composed of single (acinus) or multiple lobules that open into a duct. When associated with hair, sebaceous glands open to the pilar canal [8]. In each acinus, sebocytes are compartmentalised in 3 zones: in the periphery; the maturation zone and sebocytes of the necrotic zone (in the center). In the periphery we find a population of undifferentiated sebocytes that express cytokeratin 7 [7,8]. These cells are small, have cuboidal

shape and are in contact with the basal layer composed of keratinocytes. During maturation, sebocytes start rounding up, accumulate lipids and their nuclei become pycnotic. Fully mature cells are located in the necrotic zone. They are “ready to die” in order to release their sebum [8].

During lipid accumulation, expression of proteins involved in lipid metabolism and storage is increased. For instance, treatment of primary sebocytes with sebum-inducing compounds such as linoleic or arachidonic acids increases expression of enzymes involved in lipid metabolism such as: FATP4 (Fatty Acid Transport Protein 4), FASN (Fatty acid synthase), FADS2 (D6 desaturase) *etc*. Lipid storage proteins, such as members of the perilipin family (*e.g.* PLIN2) that surround lipid droplets, are also increased [9, 10, 11]

3D models with sebaceous glands consists in: skin explants, sebaceous gland explants or 3D skin organoids [11]. 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 [9]. 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 [11,12]. 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.

To our knowledge, two innovative 3D skin models including sebocytes have been developed so far [13, 14]. The first model consists of bioprinting hiPSC derived sebocytes into a complex matrix composed of chitosan, glycosaminoglycan and collagen [13]. The second model also uses bioprinting technology but sebocytes are isolated from biopsies [14]. In our model we use hiPSC-derived sebocytes by bioprinting spots of sebocytes in a collagen-based dermal model.

Bioprinting has become an essential tool in skin bioengineering. Bioprinting technologies can be grouped in nozzle-based technologies: micro-valve technology, bio-extrusion and Reactive Jet Impingement (REJI) and nozzle-free technologies: laser-assisted bioprinting (LAB) and sterolithography-based bioprinters. All technologies have pros and cons such as cost, reliability, speed, resolution, cell viability and handling of printed skin samples due to their size. Hybrid bioprinters combining nozzle-based and laser-assisted bioprinting have major advantages compared to the methods mentioned previously and limited drawbacks.

We therefore decided to use such multimodal bioprinters to develop a 3D skin model containing structures similar to sebaceous glands [15].

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 Methods

Rodent sebocyte cell culture

Sebaceous cell lines were cultured with 5 % CO_2 at 37°C and 90 % humidity atmosphere in regular DMEM-F12 (Gibco, USA) supplemented with: 10 % heat-inactivated fetal bovine serum (FBS, Gibco, South America), 1.05 mM Ca^{2+} (Sigma, USA), 4 mM glutamine (Gibco,UK), 10 ng/mL EGF (Sigma, USA), 100 IU/mL penicillin-streptomycin (Gibco, USA), and 1 µg/mL Amphotericin B (Gibco, USA).

For lipid production induction or inhibition cells were seeded at 20 000 cell/ well in 96 well plates. For cell treatment, products were prepared in K-SFMc medium (Gibco, USA) supplemented with: 5 % FBS (Gibco, South America), 0.09 mM Ca^{2+} (Sigma, USA), 5 ng/mL EGF (Sigma, USA), 0.6 % Bovine Pituary Extract (EPB, Gibco, USA), 100 IU/mL penicillin-streptomycin (Gibco, USA), and 1 µg/mL Amphotericin B (Gibco, USA).

Cells were treated with 10^{-4} M linoleic acid (Sigma, USA) or 10^{-3} M epigallocatechin gallate (EEGCG, Sigma, USA). After 24 h of treatment cell media was discarded and cells were fixed with histochoice (VWR, USA). For lipid visualisation cells were stained with 3mg/mL Oil Red O (Sigma, USA).

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.

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, fepidermal 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

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. Setting-up bioprinting parameters

We decided to use an easy to handle and cost-effective, rodent sebaceous cell-line. These cells have the capacity to respond to sebum-modulating stimuli: both activating (e.g.linoleic acid) or inhibiting (e.g.EGCG) (Fig1A). These cells were therefore used to set up bioprinting parameters.

Spots of several hundreds of rodent sebocytes were printed on a collagen lattice according to the pattern shown in Fig1B. Aggregates of viable cells were visible already at 24h post-bioprinting (Fig1C).

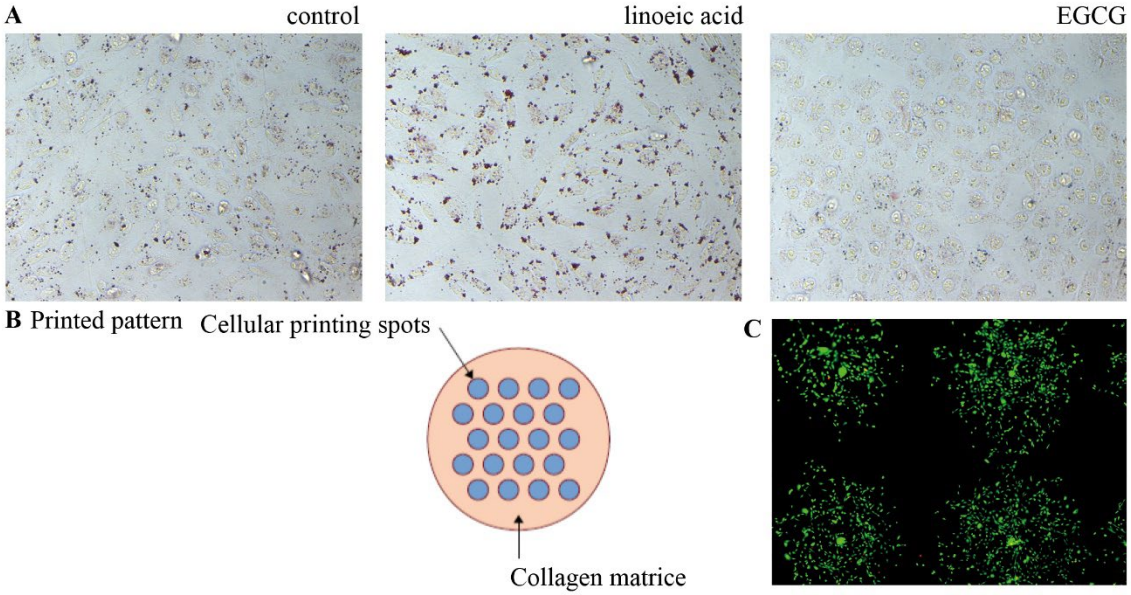

Figure 1: Bioprinting rodent sebaceous cell line. A. Effect of linoleic acid and ECGC on sebum production **B.** Bioprinting pattern **C**. Viability staining of sebocytes after printing.

We next tested the compatibility of our aggregates with media used for skin model production (Fig 2A). Models were treated with classic sebaceous growth media, with media used during skin immersion phase or with media used during skin maturation phase. Aggregates of 150-300µm of diameter were obtained in the 3 conditions (Fig 2B). To study internal organisation of cells, aggregates were cut and stained with H&E (Fig 2C). Despite the similarity with regards to aggregate size, cell organisation was modulated by the media. While in sebaceous growing media and in skin maturation media no structural organisation occured, immersion media induced a cell reorganisation.

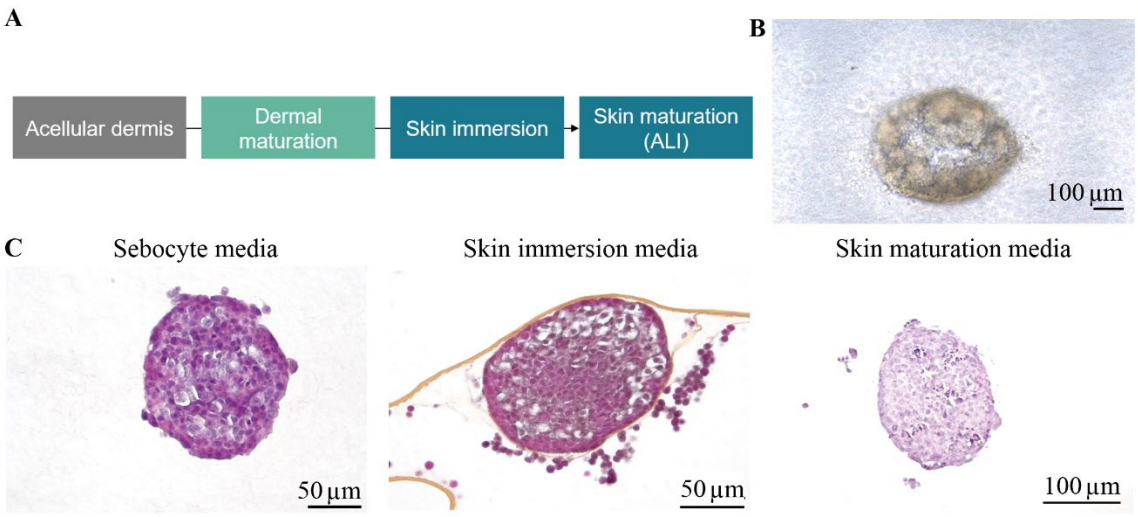

Figure 2 Aggregate compatibility with media used in skin bioprinting. A. Scheme of skin bioprinting steps (ALI: Air Liquid Interphase) **B.** Representation of an aggregate **C.** H&E staining of aggregates cultivated in different media.

Altogether these data showed that we were able to obtain viable spheroids of sebocytes that were compatible with media used during skin production. We therefore decided to pursue by printing human cells.

2. Switching to hiPSC-sebocytes

Human sebocytes were printed using parameters set up with rodent sebocytes. Similar to rodent cells, human sebocytes were also viable 24h after printing. However, we observed that aggregates of hiPSC-Seb were more dispersed, they divided less and were lost already 48h after printing (Fig 3A). 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 3B). Using these new settings, we managed to have isolated aggregates for at least 6 days.

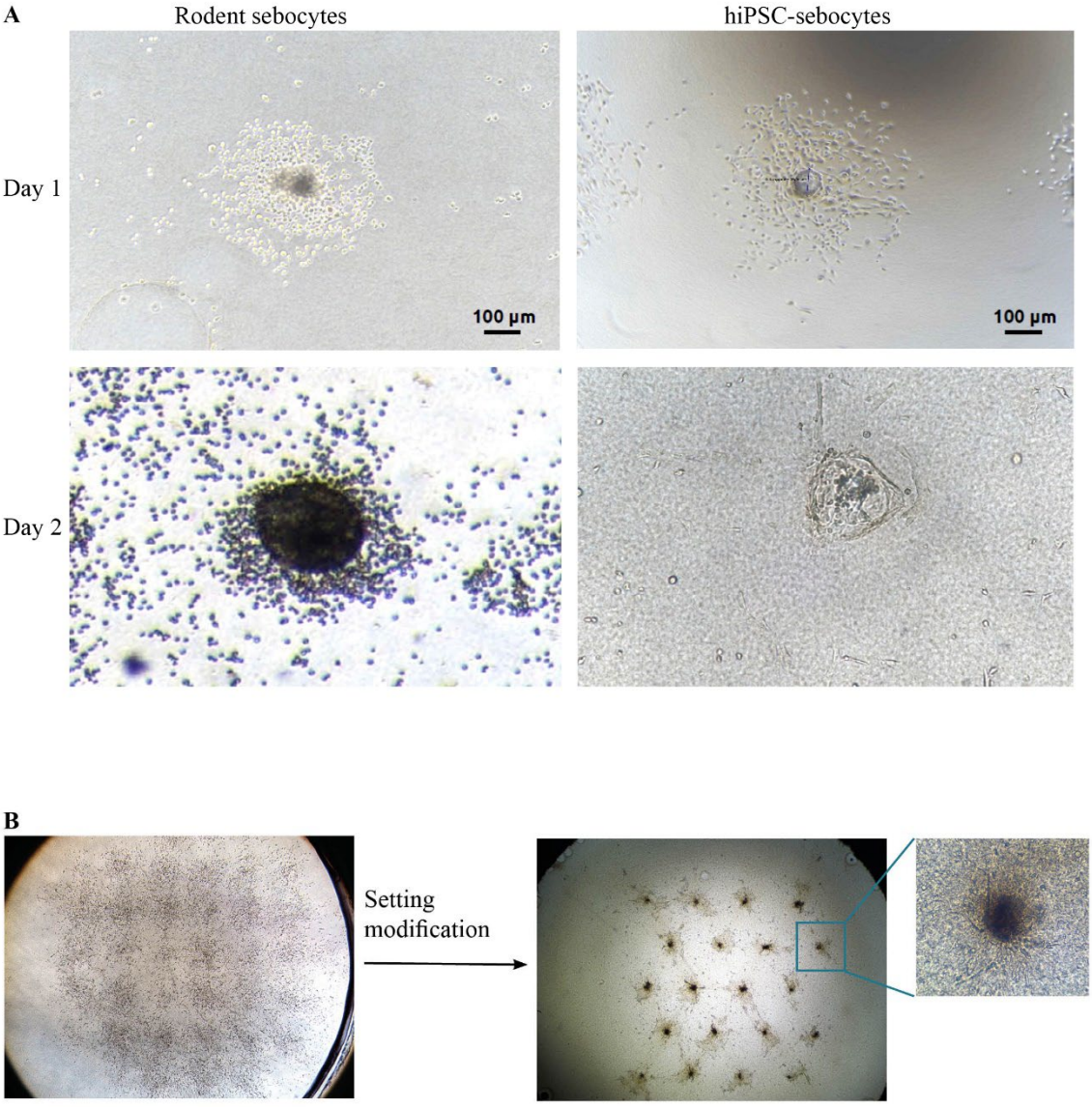

Figure 3 Bioprinting of hiPSC-sebocytes A. Loss of aggregates 48h post bioprinting **B.** Modification of bioprinting settings allowing aggregate maintenance at day 6

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 4A). 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 (Fig 4B). To solve this issue, we covered sebocytes with a layer of collagen. We could therefore keep our sebocytes for at least 11 days (Fig 4C)

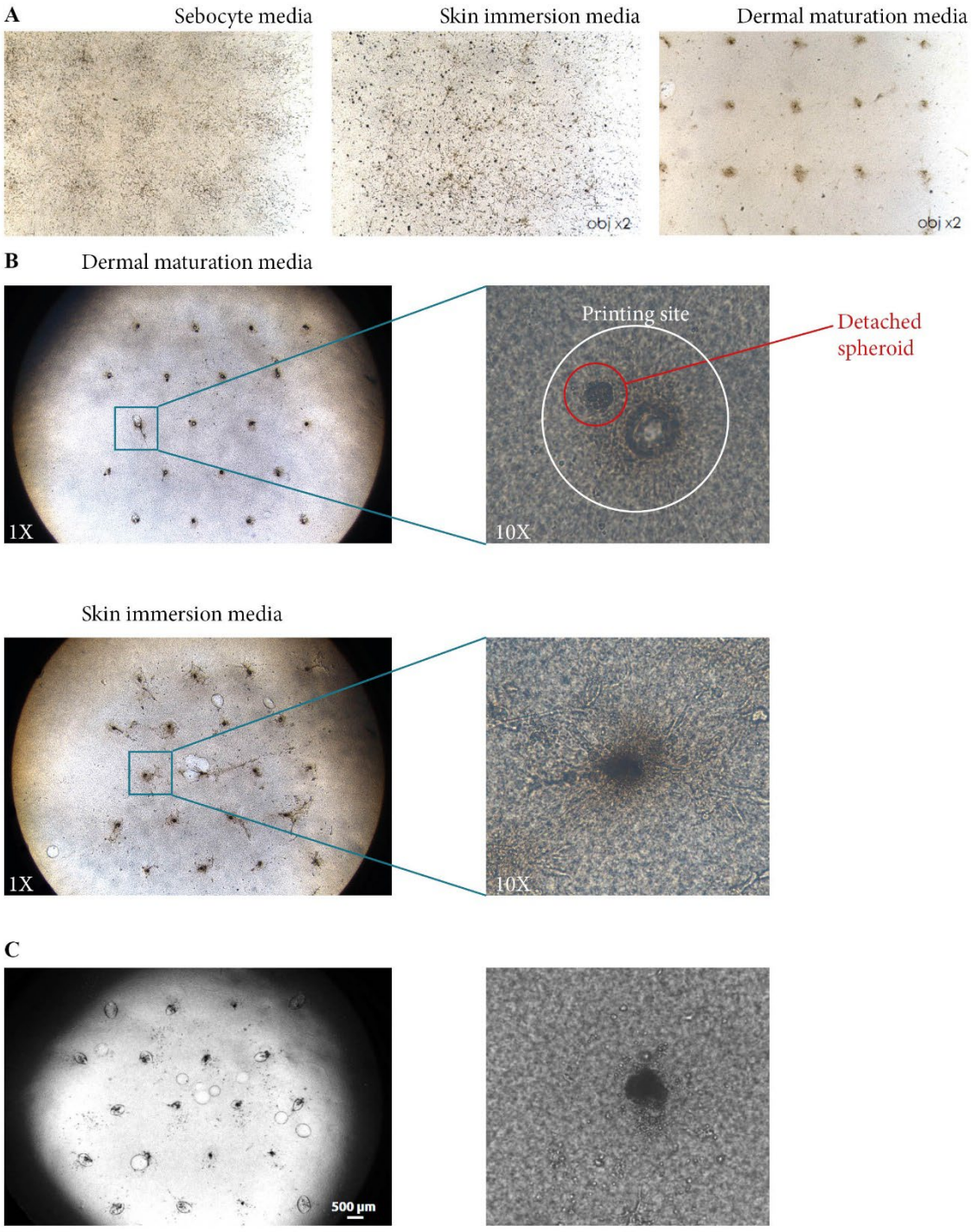

Figure 4 hiPSC-sebocyte-aggregate compatibility with media used in skin bioprinting. A. Culture with sebocyte media, skin immersion media or dermal maturation media. Maintained aggregates when cultured in dermal maturation media **B.** Sequential cultivation in dermal maturation media followed by skin immersion media **C.** Avoiding aggregate detachment by embedding in collagen gel

3. Generation of equivalent dermis containing 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 5A. Individual aggregates were maintained during dermal contraction (Fig 5B). Dermal models were cut, and morphology was studied. Sebaceous structures were visualised by Masson-Goldner trichrome staining and lipid staining (Fig 5B).

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 5C). 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.

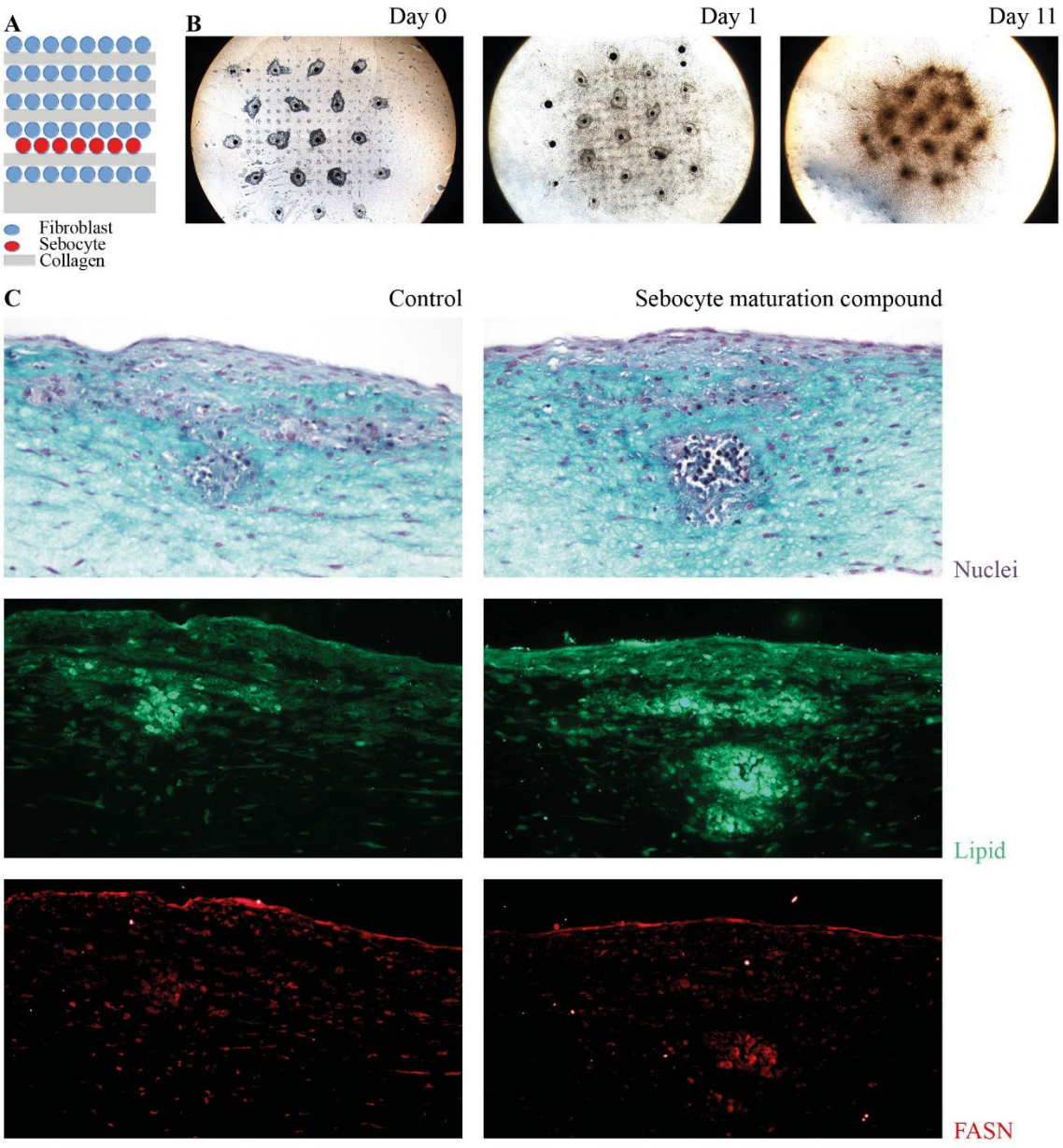

Figure 5 Generation of dermis containing sebaceous gland-like structure A. Bioprinting scheme **B.** Aggregate behaviour from 0 to 11 days after bioprinting **C.** Functional evaluation of sebaceous-like glands when treated with a compound inducing sebocyte maturation

4. Bioprinting in full skin

After having successfully embedded sebaceous gland-like structures into equivalent dermal models, we bioprinted full skin models with sebocytes as shown in Figure 6A. Surprisingly, aggregates migrated toward epidermis. We stained our models with Masson-

Goldner trichrome for histology evaluation (Fig 6B). Models were stained for lipids and visualised by light sheet microscopy (Fig 6A). Epidermal structure was however not optimal. Further studies are necessary and will focus on improvement of our model.

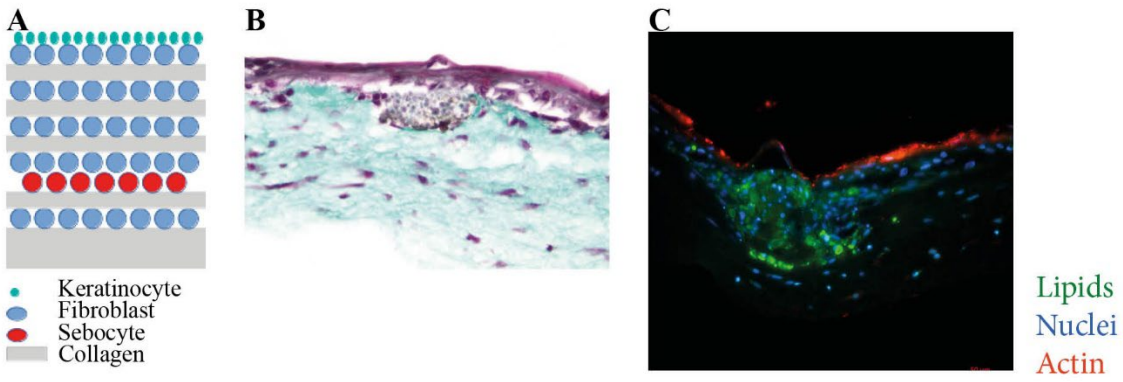

Figure 6 Generation of full-skin containing sebaceous gland-like structure A. Bioprinting scheme **B.** Skin histology, H&E staining **C.** Lipid staining visualised with light sheet microscopy

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.

Conflict of interest: None

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