3D-innervated epidermis grown on microfluidic chip

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Abstract

Background: Skin innervation contribute to skin thickness and functionality with both essential sensing and neuro-induced skin reactions. Using skin biopsies and reconstructions coupled to non-human neurons, we previously demonstrated an age-related decrease of nervous network and a lack of neuromediators leading to thinner epidermis.

This work presents an advanced human based innervated skin models using the most advanced Organ-on-Chip technologies with the aim to test neurocosmetic ingredients.

Methods: The novel Neuroskin-on-chip Technology uses a specific PDMS microfluidic device containing four chambers connected together with a large central deposition chamber for the keratinocytes perfused by another chamber located underneath and physically separated by a porous polycarbonate membrane and two peripheric channels accepting hiPSC-derived neurons connected to the central chamber by microchannels. Immunostaining on chip and image analysis were used to evaluate viability and maturation,

Results: The first results demonstrated a long-term viability until 4 weeks. Without keratinocytes, initial axonal projections were observed in the lateral microchannels after 7 days. At 21 days, neurites have grown up to 33% of the central chamber width and maturation markers were observed (peripherin, substance P, TrKB, Nav1.8). When keratinocytes were added, integration of axonal network within keratinocytes was observed.

Conclusion: We succeed to develop and grow an innervated epidermis on chip for the first time. These more physiological 3D-innervated skin models will be of real interest to screen neurocosmetic active ingredients targeting neurons and neuroskin interactions to better maintain and protect the skin from damages caused by aging or stresses.

Keywords: Microfluidic; Neuroskin; Sensory neurons, Neurocosmetic

Introduction.

Skin innervation is mandatory to maintain a healthy-looking skin thickness and functionality with both essential sensing and neuro-induced skin reactions. First proof of innervation importance for skin quality was obtained when skin denervation was shown to influence keratinocyte proliferation and therefore decrease epidermal thickness [1-2]. Then age-associated decreased epidermal innervation of facial skin was described and for these authors, age-associated decreased innervation may be the result of decreased synthesis of neurotrophic factors such as Nerve Growth Factor [3]. Since then, the cosmetic industry is focusing on discovering neurocosmetic functional ingredients that could improve the interactions between the skin and the nervous system to reduce skin aging or skin stress signs. In our laboratory, we first developed an innervated skin model made of skin biopsy cocultured with neurons. Advantageously this reinnervated skin model was used to demonstrate the clear benefits of skin reinnervation [4], and that an active ingredient could even improve epidermal cell proliferation, epidermis thickness and cohesion but also decrease cell apoptosis [5].

However, this model did not perfectly reflect the skin physiology as the neurons were coming from animal dorsal root ganglions (DRG), the neuronal bodies were located within the dermis and the skin was not a reconstructed one but was a skin biopsy with a limited shelf-life in good conditions. Several improvements had to be made, at first the use of human derived sensory neurons, the delocalization of neuronal bodies from the skin model itself and the use a real living skin. Thus, our goal was to combine the use of human induced pluripotent stem cells (hiPSC) to generate sensory neuron progenitors and the most advanced Organ-on-Chip technologies to first produce and grow an innervated epidermis on chip, and later an innervated skin on chip.

Cosmetic industry decided to ban animal testing since 2004, thus companies needed to develop alternative models to prove the efficacy of their active ingredients. Since then, various strategies have been used. Primary cells from surgical waste are widely used as well as cell lines when primary cells are hard to culture such as sebocytes or not available such as

neurons, with the advantage to obtain a high number of cells. However, some trends linked to well-being or inflammation mediated by sensory neurons of the skin could not be addressed anymore. In 2006, Takahashi & Yamanaka published the first article about how to obtain pluripotent cells from somatic cells [6]. Mouse embryonic and adult fibroblasts were dedifferentiated using defined factors Oct3/4, Sox2, c-Myc and Klf4. These cells grafted subcutaneously into nude mice resulted in tumor containing a variety of tissues from all three germ layers. Since then, the scientific community has used induced pluripotent stem cells (iPSC) to obtain skin cells close to primary cells. In 2013, Itoh *et al*. published for the first time a work on 3D skin equivalent with fibroblasts and keratinocytes derived from human iPSC [7]. First iPSC derived neurons were obtained for medical use [8-9] and later a reconstructed skin with human iPSC-derived sensory neurons has been produced [10].

In order to increase screening capability, better reproduce physiology and to mimic a more dynamic nutriments supply, cell culture also turns to miniaturization and microfluidic, giving birth to organ-on-a-chip models. The organ-on-a-chip is an intriguing scientific and technological development in which biology is coupled with microtechnology to mimic key aspects of human physiology. The chip takes the form of a microfluidic device containing networks of hair-fine microchannels for guiding and manipulating minute volumes (picolitres up to milliliters) of solution. The organ is a more relatable term that refers to the miniature tissues grown and residing in the microfluidic chips, which can recapitulate one or more tissue-specific functions. Although they are much simpler than native tissues and organs, scientists have discovered that these systems can often serve as effective mimics of human physiology and disease [11-12]. Keratinocytes [13] and later skin-on-chip model have been developed with epidermis and dermis [14-15]. Some works have studied nociception using rodents dorsal root ganglions [16] hippocampi neurons from rat [17], or with keratinocytes [18]. Recently an interesting work about a neurosensory-epithelial interaction presents the link between iPSC-derived neurons and keratinocytes [19] but until now, none adress the full-human a 3D innervated epithelial structure in the microfluidic field.

For this study, we developed a novel Neuroskin-on-chip Technology with the aim to:

1. integrate human induced pluripotent stem cell (hiPSC)-derived sensory neurons in a growing 3D epidermal reconstruction
2. validate each biological functions independently
3. evaluate the performance of such model using modulators
4. then to test and select neurocosmetic active ingredients.

Compared to existing innervated epidermis or skin models made by classical coculture with or without insert, this model holds the promise to better mimic the real skin innervation physiology. Indeed, neuronal bodies will be fluidly isolated from the growing skin model thanks to microfluidic channels induced compartmentalization. Such connecting microchannels allow sprouting of axons from one compartment to the other, which opens innervation by terminal endings in the growing skin compartment.

Materials and Methods.

Device fabrication

The microfluidic devices were fabricated according to NETRI's knowhow. Briefly, SU-8 molds were fabricated using conventional photolithography techniques, using SU-8 photoresist series. Silicon wafers were diced and integrated into 3D printed molds to create master molds. Master molds were replicated using dedicated multiple silicone and polyurethane positive replicate. Polydimethylsiloxane (PDMS, Sylgard 184, Dow Corning) was prepared and cast onto the replicated molds before being cured at 65 °C for 2h. The PDMS was then cut at the desired size, before being peeled off the mold. The inlet and the outlet zones were then punched out and the PDMS was cleaned and protected from undesired dusts exposure. The final devices were made of a microporous polycarbonate membrane (PC, IpPore) sandwiched between two microstructured layers of pretreated PDMS and assembled with on polystyrene petri dish and vacuum stored under UV exposer light for 30 minutes for sterilization purposes. Once completed, the entire stack was vacuum sealed for storage.

Culture of human iPS-derived sensory neurons, keratinocytes and epidermal reconstruction

Human-derived materials were preserved and handled with the approval and under the guidelines of French legislation.

Experiments were carried out with human iPSC-Sensory Neuron Progenitors (Axol Bioscience Ltd). Sensory neurons were seeded by placing 15 µL of a $6.5.10^6$ cells/mL neuron suspension in the inlet reservoir. Cell culture media (Sensory neuron maintenance medium with sensory maturation maximizer supplement adding NGF, GDNF, BDNF, NT3) was replaced every 2 to 3 days. Neurons were maintained in culture for up to 28 days under controlled environment (37°C and 5% CO_2).

For epidermis generation, normal human keratinocytes (breast, 20y/o female) were used and grown in Epilife medium (Gibco) supplemented with 1.5mM calcium (VWR Chemicals), HKGS (Gibco) and then 10ng/mL KGF (Sigma-Aldrich) and 50 µg/mL ascorbic acid (Sigma-Aldrich).

Immunostaining protocol & analysis

Cultures were fixed in 4% paraformaldehyde (PFA) for 30 minutes at room temperature. Cells were washed three times with PBS and permeabilized for 10 minutes with 0.1% Triton-X100 followed by 30 minutes with 3% BSA.

For neuron characterization, primary antibodies (Nav1.8, Peripherin and NF160 Abcam, substance P Invitrogen, TrkB Origen, B-III tubulin Biolegend) were then added, and the devices incubated overnight at 4°C. For epidermis characterization, primary antibodies cytokeratin 10 and filaggrin (Abcam) were used

The cells were then rinsed three times with PBS and further incubated with the corresponding secondary antibodies for 2 hours at room temperature. Images were acquired with an inverted epifluorescence microscope AxioObserver 7 (Zeiss) fitted with a CMOS camera. Images were routinely processed using FiJi software (imageJ 1.46). Raw images were stitched using the stitching plugin of FiJi.

In order to selectively count cells, three random areas on each image were first converted to mask using imageJ's thresholding routing, before applying a watershed treatment. The resulting binary image was then analyzed using the "Analyze Particles" innate imageJ's function, using a size and a circularity filter (objects smaller than 7 µm, bigger than 20 µm or with a circularity smaller than 0.1 were excluded from analysis). The resulting contour image, the merge of the contour with a duplicate of the original image and the associated data (number of objects, mean area, % of coverage, etc.) were then saved.

Results.

Because of the uniqueness of the microfluidic design, we created a specific PDMS microfluidic device containing four major chambers connected together (Figure 1). First, in the center of the chip, a 64mm² large central deposition chamber had the capacity to accept primary keratinocytes. Second, two peripheric channels accepting hiPSC-derived neurons were place sideways of the central deposition chamber and were both connected to the central chamber by microchannels. Finally, the central chamber could be perfused by another chamber located underneath and physically separated by a porous polycarbonate membrane. Such compartmentalization opens perfusion capacity of the central upper chamber by the lower one using different culture media supplemented with specific additives allowing neuronal or epidermis maturation.

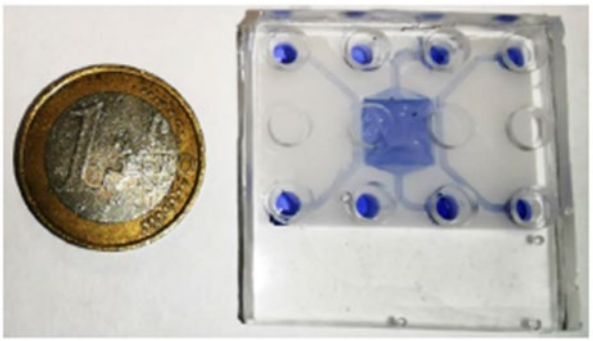

Figure 1: Picture of the microfluidic device filled with blue ink.

The choice of the supplier of human sensory neurons derived from hiPSCs was made in 3 steps. A listing was made taking into account industrial suppliers of cells, companies specialized in the reprogramming and differentiation of hiPSCs cells and academic partners. Cells from academic partners were excluded due to reproducibility difficulties between cell batches as the process of cell generation that would have been too time-consuming. Among two pre-selected industrial suppliers, Axol Bioscience was used as a Maximiser medium could be additionally used, and more functional characterization had been made with their hiPSC-Sensory Neuron Progenitors. Finally, the culture out of chip and immunostaining of

specific markers according manufacturer recommendations allowed to validate the source of hiPSC-Sensory Neuron Progenitors.

When used on the chip without keratinocytes in the central chamber, the first results demonstrated a long-term viability until 4 weeks with a decrease of Nestin/Sox2 at 22 days *in vitro* of hiPSC-derived sensory neurons, indicating a proper differentiation process directly within the microfluidic device. We observed after 7 days *in vitro* specific morphology of human sensory neurons in vitro with initial axonal projections in the lateral microchannels.

After 21 days in vitro, maturation markers contributing to neuronal functionality were observed and quantified (Figure 2). Structural Peripherin (PRPH) intermediate filament mainly expressed in neurons of the peripheral nervous system and Class III β tubulin (B-III tubulin) expressed in post-mitotic neuron cytoskeleton where highly expressed. TrkB, the high affinity receptor for BDNF, NT-3, NT-4/5 promoter of neuronal survival, substance P peptidergic nociceptor involved in neurogenic inflammation correlated to neuronal maturation and Nav1.8 which play a role in pain at low temperature were expressed to a lesser degree.

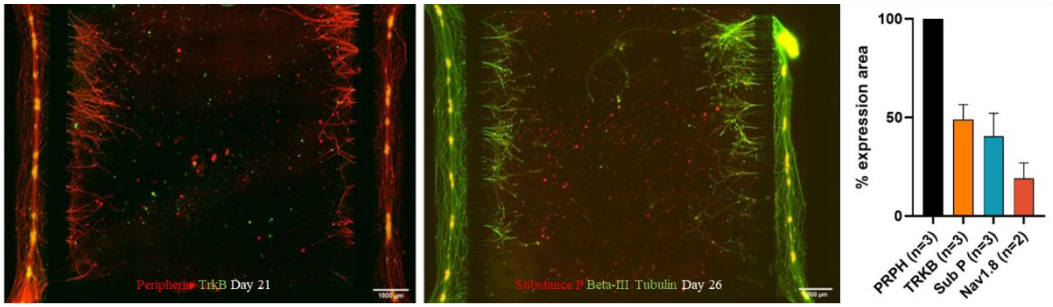

Figure 2: Immunofluorescence pictures of human sensory neurons in microfluidic device and quantification by image analysis after 21 days of culture.

To further characterize the human sensory neurons in the microfluidic device, a measurement of the longest extensions observed in the central chamber was performed. As shown in Figure 3, after 21 days in vitro, neurites have grown from both side and up to 33% of the central chamber width (2.4 mm or 3.33mm including 450µm corresponding to the size of the microchannels between the lateral channel and the central chamber).

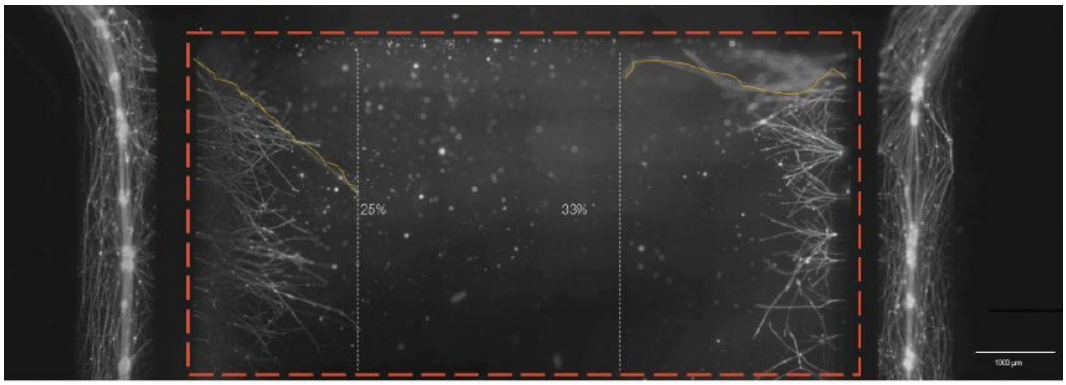

Figure 3: Representative image of innervation extensions measured by segmented line at day 21 (dotted line equivalent to the longest extension observed and expressed as percentage of the of chamber width).

When keratinocytes were grown, some retractation was observed for the epidermal layer at the borders. Regarding the integration of axonal network within keratinocytes, few connections between keratinocytes were seen. The epidermis is ongoing differentiation as shown by few cells that began to express filaggrin in the upper layer.

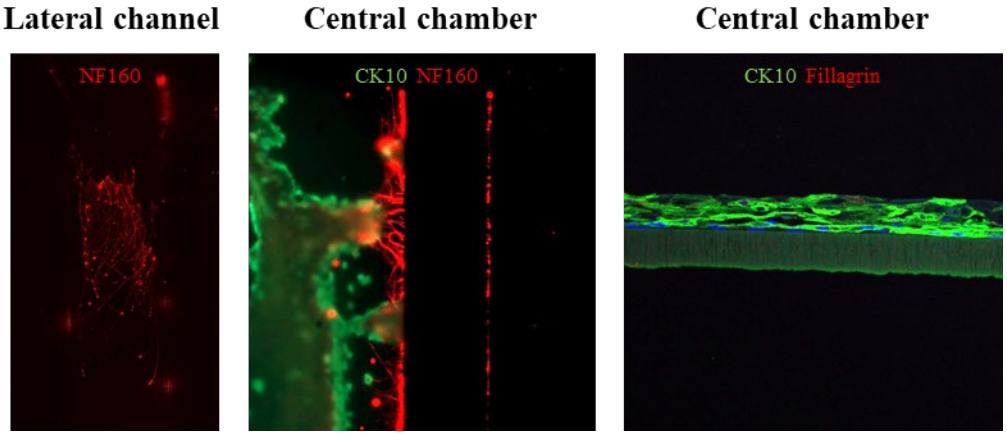

Figure 4: Representative image of cocultured neurons and keratinocytes, in the lateral channels and in the chamber.

Discussion.

We manage to cultivate on chip sensory neurons from hiPSC with initial axonal projections in the lateral microchannels from 7 days. At 21 days, sensory neurons could colonize the central chamber from both sides, they could grow their axonal projections up to the 2/3 of the central chamber in 21 days and they expressed the expected differentiating markers Substance P and Nav 1.8.

When keratinocytes were added in the central chamber at 13 days of the preliminary neuron culture, within our first experiments we observed less longer neuronal extension in the central chamber compared to the condition without keratinocytes. Even keratinocytes were seeded everywhere in the central chamber, we observed a retractation of the epidermal layer on the borders, however some connections between axons and keratinocytes were observed. Regarding the epidermis, up to 5 superposed layers could be observed with a beginning of differentiation observed by filaggrin expression.

However, the epidermis part still needs to be improved and we thought to first improve the coating or the seeding/proliferation step to get a basal layer with a more palisadic organization. To obtain a ticker epidermis, we plan to have a longer period of culture and a better connection between neurons that we expect to obtain by the addition of nerve growth factor in the culture medium of keratinocytes to favor the neuronal extension even in the presence of keratinocytes. Finally, the drying of the central chamber has also to be improved to get a better the differentiation of the epidermis.

Conclusion.

We succeed to develop and grow an innervated epidermis on chip for the first time.

Pharmaceutical proof of concept will be later done by neuropeptide releases quantification (substance P, calcitonin gene-related peptide …) and epidermal thickness measurement. Ultimately, the model would be improved by innervating and growing a full skin model.

These more physiological 3D-innervated skin models will be of real interest to screen neurocosmetic active ingredients targeting neurons and neuroskin interactions to better maintain and protect the neuroskin integrity from damages caused by aging or stresses.

Conflict of Interest Statement. NONE.

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