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In vitro activation of the neuro-transduction mechanism in sensitive organotypic human skin model

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ABSTRACT

Recent advances in tissue engineering have encouraged researchers to endeavor the production of fully functional three-dimensional (3D) thick human tissues *in vitro*. Here, we report the fabrication of a fully innervated human skin tissue *in vitro* that recapitulates and replicates skin sensory function. Previous attempts to innervate *in vitro* 3D skin models did not demonstrate an effective functionality of the nerve network. In our approach, we initially engineer functional human skin tissue based on fibroblast-generated dermis and differentiated epidermis; then, we promote rat dorsal root ganglion (DRG) neurons axon ingrowth in the de-novo developed tissue. Neurofilaments network infiltrates the entire native dermis extracellular matrix (ECM), as demonstrated by immunofluorescence and second harmonic generation (SHG) imaging. To prove sensing functionality of the tissue, we use topical applications of capsaicin, an agonist of transient receptor protein-vanilloid 1 (TRPV1) channel, and quantify calcium currents resulting from variations of Ca^{++} concentration in DRG neurons innervating our model. Calcium currents generation demonstrates functional cross-talking between dermis and epidermis compartments. Moreover, through a computational fluid dynamic (CFD) analysis, we set fluid dynamic conditions for a non-planar skin equivalent growth, as proof of potential application in creating skin grafts tailored on-demand for *in vivo* wound shape.

1. Introduction

Tissue engineering holds the promise to recreate fully functional 3D thick human tissues *in vitro* for regenerative medicine and tissue on-chip applications [1,2]. Fully functional *in vitro* engineered 3D human skin has been recently reported by our group [3] proving that, by correctly recapitulating morphogenic development along with synthesis and reassembly of native ECM, it is possible to produce functional skin tissues including follicle appendages. An important aspect that remains to be addressed is to enrich the functionality of these bio-hybrid tissues by adding the *tactility* and *sensitivity* functions through the innervation of the tissue. Sensory nerve fibers from the DRG innervate the dermis and the base of epidermis [4–6]. The dermis provides guidance cues to axons growth and, at the same time, the specific cell-cell and cell-ECM interactions occurring in it direct the migration of axons to their targets. The epidermis, particularly keratinocytes and Merkel cells, represents the forefront of the sensory system [7–9]. Epidermal cells express many receptors, neuropeptides and sensorial proteins that interact with sensory nerve cells [10]. Among them, TRPV1 is the most characterized sensor protein and is highly expressed within the epidermis and in the neurons involved in pain or pruritus transmission and neurogenic inflammation [6,10,11]. According to these natural features, human skin is remarkably able to sense external stimuli producing appropriate feedbacks that, through a mechanism of sensory transduction, reach the central nervous system and acting as a peripheral neuro-endocrine organ [12,13]. The high incidence of skin injuries can lead to loss of peripheral nerve sensory function and involve different parts of human body, such as mainly hands or fingers that need to be regenerated, especially when amputations are not be possible. Such need has pushed researchers to get an *in vitro* innervated system to restore the damaged functions [14,15]. Several *in vitro* 3D systems

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have been developed for culturing neurons in an environment that mimics the *in vivo* conditions. However, most of the *in vitro* innervated skin models investigated on the neuritis growth and their influence on the other skin cell types rather than on the sensory function of the innervated skin model [16–24]. Gingras et al. developed and characterized a tissue-engineered skin model of peripheral nerve regeneration by using collagen sponge populated with human endothelial cells and/or human fibroblasts in order to study neurite outgrowth and assess the influence of endothelial and epidermal cells on neurite growth [25,26]. Subsequently, Cadau et al. used the same model as innervated tissue-engineered skin to investigate the impact of glycation and to screen anti-advanced glycation end-products [27]. Despite such models resulted very interesting in mimicking the *in vitro* peripheral nerve regeneration process, studies on neuronal sensory functionality are still limited to *ex vivo* re-innervated human skin explants [28–32]. Since the cell-ECM interactions have a fundamental role in providing the correct guidance to axon outgrowth, in alternative to the use of *ex vivo* explant, several strategies that induce the production of tissue equivalent, where cells are embedded in their own ECM, seem the more promising ones to obtain an *in vitro* fully functional tissue [33–38]. Indeed, the quality of most of current human skin equivalent (HSE) models is often compromised by their largely non-human dermal equivalents origin (e.g. collagen originating from rat or bovine tendon, and bovine fibrin), which makes dermal compartment an incomplete approach to human ECM *in vivo*, leading to a model that scarcely recapitulate the functionality of its *in vivo* counterpart. Here, starting from a previously developed tissue engineering strategy to induce *in vitro* tissue genesis of skin [39–43], we developed for the first time an *in vitro* innervated organotypic human skin model able to perform and replicate skin sensory function. The first aim of this work focused on the assessment of our innervated organotypic model capability to reproduce native skin sensory transduction mechanism in response to capsaicin in generating Ca^{++} currents. Furthermore, we developed a non-planar HSE tissue as proof of the possibility to create skin grafts tailored for fitting to a specific wound shape with our strategy. These results prove that sensitivity functions of human tissues can be reproduced *in vitro* and open the way to use these tissues both *in vitro,* as testing platform for studies on signal transmission and for screening of biomolecules acting on the sensory neurons, and *in vivo,* as a new class of living prosthetic biomaterials.

2. Materials and methods

2.1. Dermal formation

Human dermal fibroblasts (neonatal HDF 106-05n ECACC) were sub-cultured onto 150 mm Petri dishes in culture medium (Eagle's BSS Minimum Essential Medium containing 20% Fetal Bovine Serum, 100 mg ml^{-1} L-glutamine, 100 U ml^{-1} penicillin/streptomycin, and 0.1 mM Non Essential Amino Acids) (Lonza and Sigma Aldrich Chemical Companies). Cells were maintained at 37 °C in humidified atmosphere containing 5% CO_2. HDFs at passages 6 were used for experiments. Cross-linked porous gelatin microbeads were prepared according to a modified double emulsion technique (O/W/O) [41], sterilized by absolute ethanol, washed in phosphate buffer solution (PBS) and cultured with HDFs in a spinner flask for 7 days until human dermal-microtissue precursors (HD-μTPs) formation, as previously described [39]. The growth medium was changed on the 1st day and every 2 days until the end of experiment. From the 2nd day, 50 μg ml^{-1} of ascorbic acid were added. HD-μTPs suspension was transferred by pipetting into the maturation chamber to allow their molding in different 1 mm-thick planar dermal equivalent (6 × 8mm, 50 × 20 mm and 17 mm in diameter shapes). During the filling procedure, no bubble formation was assured into maturation chambers, which were closed, placed on the bottom of a spinner flask and completely covered by culture medium. The spinner worked at 60 rpm and the medium was exchanged every 3 days, adding 50 μg ml^{-1} of ascorbic acid at every medium exchange. After 2 weeks of culture, rectangular-shaped (50 × 20 mm) and disk-shaped (17 mm in diameter) dermis equivalent were fitted inside a cylindrical stainless steel net mold (13 mm-internal diameter), previously on purpose welded, in order to allow the formation of a phalanx-like dermal equivalent shape. The disk-shaped dermal equivalent was lying on top. They were left in culture for 20 days until complete assembly of dermal equivalent in the desired shape.

2.2. Epidermal formation

The immortal human keratinocyte line of HaCaT cells (passages 20–30) were grown in Keratinocytes Basal Medium 2 (KBM2) containing 0.004 ml ml^{-1} Bovine Pituitary Extract (PBE), 0.33 ng ml^{-1} hydrocortisone, 10 -μg ml^{-1} of transferrin, 0.125 ng ml^{-1} h-EGF, 5 μg ml^{-1} insulin, 0.39 μg ml^{-1} epinephrine, 0.06 mM $CaCl_2$ and 100 U ml^{-1} penicillin/streptomycin) (Lonza). Cells were maintained at 37 °C in humidified atmosphere containing 5% CO_2. HaCaT cells were seeded both on planar and on phalanx-like dermal equivalent in submerged and air-liquid cell cultures. Regarding planar dermis equivalent, after 3 rinsing with PBS and few minutes of drying in sterile condition, each planar construct was put on a transwell and covered with human fibronectin (50 μg ml^{-1}) (Sigma Aldrich). Cell seeding (3.5 10^5 HaCaT cells/each construct) was carried out and, then, followed by medium filling. The planar dermis equivalent were maintained for 1 week in submerged culture condition at 37 °C in humidified atmosphere containing 5% CO_2 and reducing the percentage of FBS in Keratinocytes Growth Medium 2 (KGM2, from Lonza) from 5% to 0% at each change. Then, the planar dermis equivalent was cultured in air-liquid conditions for further 2 weeks with medium exchanges every 2 days. Regarding HaCaT cells seeding on phalanx-like dermal equivalent, submerged and air-liquid cultures were carried out by using two different bioreactor systems. A 0.3 mm-thick porous poly-L-Lactic acid (PLLA, from Evonik Industries) cylindrical support with round top was used to facilitate the dermal equivalent transfer. The polymeric film, that was fabricated according to a modified technique of a previous work [44], was detached from the stainless steel support (external diameter = 15 mm), rinsed in distilled water, put in 70% ethanol for 2 h and, then, in cell culture medium before using. The bioreactor system for submerged HaCaT cells culture was designed by means of CAD software (Creo Parametric) and consisted of Teflon and stainless steel modules, opportunely assembled. Phalanx-like shape dermal equivalent fitted on PLLA support was insert on Teflon module, rinsed with PBS, dried for few minutes in sterile condition and conditioned with fibronectin (50 μg ml^{-1}) (Sigma Aldrich Chemical Company) for 45 min. Then, HaCaT cells (4 10^5 in 5 μl of KGM2 for each step) were seeded on it all along the length of the dermal equivalent including the top. The seeding process consisted of 4 steps, each one every quarter of rotation of the dermal equivalent. Each seeding step lasted half an hour and, then, all the system was put inside a glass chamber and maintained at 37 °C in humidified atmosphere containing 5% CO_2. Drops of KGM2 were added to hydrate the dermal equivalent every 15 min and, at the end of the last half an hour, the bioreactor was filled with HaCaT cells medium. After 3 h the sample was collected for morphological analysis. The system worked in dynamic conditions for 7 days by means of a gentle magnetic stirring and the medium was fulfilled every 3 days,