Design of an Integrated Microvascularised Human Skin-on-a-Chip Tissue Equivalent Model

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Abstract

Tissue engineered skin constructs have been under development since the 1980s as a replacement for human skin tissues and animal models for therapeutics and cosmetic testing. These have evolved from simple single cell-assays to increasingly complex models with integrated dermal equivalents and multiple cell types including a dermis, epidermis and vasculature. The development of micro-engineered platforms and biomaterials has enabled scientists to better recreate and capture the tissue microenvironment in vitro, including the vascularization of tissue models and their integration into microfluidic chips. However, to date, microvascularised human skin equivalents in a microfluidic context have not been reported. Here we present the design of a novel skin-on-a-chip model integrating human derived primary and immortalized cells in a full thickness skin equivalent. The model is housed in a microfluidic device, in which a microvasculature was previously established. We characterize the impact of our chip design on the quality of the microvascular networks formed and evidence that this enables the formation of more homogenous networks. We developed a methodology to harvest tissues from embedded chips, after 14 days of culture, and characterize the impact of culture conditions and vascularization (including with pericyte co-cultures) on the stratification of the epidermis in the resulting skin equivalents. Our results indicate that vascularization enhances stratification and differentiation (thickness, architecture and expression of terminal differentiation markers such as involucrin and transglutaminase 1), allowing formation of more mature skin equivalents in microfluidic chips. The skin-on-a-chip tissue equivalents developed, thanks to their realistic microvasculature, may find application for the testing efficacy and safety of therapeutics delivered systemically, in a human context.

1 Introduction

Our ability to recreate more complex tissue structure and function has considerable improved. As the fields of micro-engineering and biomaterials science developed, it has become increasingly clear that recreating some of the 3D architecture of tissues may be essential to reproduce the cell microenvironment and direct cell phenotype and tissue structure or function. Dating back from the 80s, micro-engineered platforms allowed the control of single cell adhesion and patterning, in turn allowing to control their phenotype (Watt et al., 1988, Chen et al., 1997, Connelly et al., 2010, Tan et al., 2013). More recently, the formation of clusters allowing to capture more complex behaviors, including cell segregation, motility, sprouting and differentiation have been developed (Nelson et al., 2006, Ruiz and Chen, 2008, Gautrot et al., *2012,* Costa et al., 2014). However, these platforms remained inherently based on 2D models, certainly relevant to cells forming 2D assemblies such as epithelia, but clearly limiting the creation of more complex architectures. Although interesting strategies were proposed to bridge the 2D and 3D world (Bosch-Fortea et al., 2019), making use of 2D patterns to guide cell-assembly in a 3D matrix, our ability to micro-engineer 3D tissues required the development of novel platforms.

The field of skin biology was certainly the first to see striking progress in the development of complex hierarchical tissue structures, with stratified differentiated skin equivalents of human tissues forming from epidermal keratinocytes assembling as monolayers at the surface of hydrogels encapsulating fibroblasts (Shahabeddin et al., 1990, Stark et al., 2006). This allowed not only the recreation of well-differentiated human skin equivalents, but also allowed capturing some of the skin barrier functions, from trans-epithelial resistance to the diffusion of therapeutics and nanomaterials (Ackermann et al., 2010, Asbill et al., 2000, Bellas et al., 2012, Mazlyzam et al., 2007). These include models which have integrated other *in vivo* skin structures and cell types including the hair follicle (Langan et al., 2015) and melanocytes (Li et al., 2011, Liu et al., 2007). More recently, a broad range of tissues have beneficiated from advances in stem cell biology, spheroid and organoid models and progress in biomaterials and microfabrication. This ranges from the formation of functional muscle and lung tissue models (Huh et al., 2010, Ronaldson-Bouchard et al., 2018, Sakar et al., 2016) to the development of complex organoid models, for example of gut, skin and brain tissues (Lancaster et al., 2013, Lee et al., 2020).

As tissues grew in complexity and size, vascularization became an important hurdle, either to their further development or growth, or simply their long term survival and applicability (Baish et al., 2011, Coloma et al., 1997, Colton, 1995). In addition, recapitulating the complex architecture and function of microvascularised tissues will be important to the accurate prediction of therapeutics safety and efficacy (Loskill et al., 2021) for example mimicking systemic delivery to targeted tissues. To address this issue, a range of tissue models have been placed in parallel with endothelialised structures, therefore allowing to recreate the endothelium-tissue interface. This has in particular be exploited in a broad range of tissue-on-chip models, using porous membranes to separate an endothelial compartment from a tissue compartment (Hassell et al., 2017, Huh et al., 2010, Kilic et al., 2016, Kim and Ingber, 2013). In addition to simply structuring tissues, this approach enables the mechanical stimulation of resulting tissues, an important factor regulating cell and tissue biology and response to pathogen or during disease progression. In order to recreate more functional models of vessels, a range of platforms have been engineered to allow the endothelialization of micro-capillaries and microchannels, allowing to study phenomena such as sprouting and angiogenesis in more realistic scenarios (Liu et al., 2021, Menon et al., 2017, Trappmann et al., 2017).

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Increasingly, microvascularised models have become popular, as enabling to accurately capture the architecture and function of microvascular networks perfusing tissues (Polacheck et al., 2014). Early work from Kamm and co-workers and Jeon et al. showed that microvascular networks can conveniently be assembled and maintained in microfluidic chips, allowing the formation of perfusable capillaries (Galie et al., 2014, Kim et al., 2013). Such models can be derived through angiogenesis, from endothelialised microchannels interfaces with hydrogel compartments separated by micro-posts, or by direct assembly of networks via vasculogenesis, therefore enabling faster formation of functional structures. Subsequently, hydrogel compartment were loaded with stem cells and disease cell lines in order to recreate vascularized tissue models (Chen et al., 2017, Jeon et al., 2015, Kim et al., 2015a). Another attractive alternative proposed consists in placing spheroids, and in some cases organoids within wells designed within the hydrogel compartment, therefore enabling the vascularization of resulting tissue models (Homan et al., 2019, Ko et al., 2019a, Shin et al., 2021, Sobrino et al., 2016). Although this remains moderately successful for the interpenetration of organoids, this strategy has been particularly promising for spheroid models.

The field of skin biology has seen the development of a range of skin equivalents interfaced with endothelial models. For example, endothelial barriers were formed at the bottoms or back of dermal compartments of human skin equivalents (Kwak et al., 2020, Lee et al., 2017, Wufuer et al., 2016) including in microfluidic chips, and micro-capillaries were allowed to assemble at the bottom of dermal constructs in transwell systems (Black et al., 1998, (Schurr et al., 1999) Such models of skin vascularization displayed improved epidermal barrier function (Kim et al., 2019, Kwak et al., 2020) and basal cell proliferation and differentiation (Kim et al., 2019), compared to non-endothelialised equivalents. However, few successful vascularized skin-equivalent models, integrated into microfluidic chips, have been reported to date.

In this report, we present the design of a full thickness human skin-on-a-chip model displaying a stratified differentiated epidermal compartment, assembled above a microvascularised dermal compartment (Figure 1). We first design a novel microfluidic chip featuring a circular central chamber separated from circumferential channels by micro-posts and characterize the impact of such structure on the morphology of the microvasculature. We then first identify culture conditions suitable for the formation of a stratified human skin equivalent in the resulting chips, prior to evaluating the impact of vascularization of the resulting tissue structure and epidermal differentiation. In addition, we introduce pericytes in the vascular compartment, in order to stabilize associated structures and evaluate the impact of such co-culture on the resulting vascularized skin equivalents. Overall, our results demonstrate the formation of vascularized skin-on-chip models that could be scaled and parallelized in order to design high throughput platforms for the testing of safety and efficacy of therapeutics.

2 Materials and Methods

2.1 Chip Design and Manufacture

Chips were designed in AutoCAD by AutoDesk and printed on acetate sheets by Micro Lithography Services, Chelmsford, UK. Microfluidic chips were generated using Sylgard™ 184 PDMS and glass coverslips, following literature protocols (Jenkins, 2013). In short, masters were manufactured by coating clean silicon wafers with a negative photo resist (SU-2050) using a spin coater set to 500 rpm for 10 seconds (ramp 100 rpm/second for the initial 5 seconds) to distribute the photoresist, followed by a spin speed of 1700 rpm for 30 seconds (ramp 300 rpm/second for the initial 4 seconds) to spread the resist to a uniform depth of 80 µm. After an initial soft bake at 65°C for 3 min and then at 95°C for 10 min to harden the surface of the photoresist, photomasks corresponding to the desired chip micro-

123 architectures were placed on the surface of the wafer and photocured with UV light at 90 mW/cm^2 for
124 2.4 s, allowing resist crosslinking to occur. Resists were post-baked at 65°C for 3 min and then at 95°C
125 for 10 min to assist the crosslinking, allowing to rest in between, then immersed in propylene glycol
126 methyl ether acetate (PGMEA, Sigma Aldrich, 484431) for 5 minutes to dissolve un-crosslinked
127 photoresist. Masters were immediately rinsed in isopropanol to remove excess PGMEA and dried
128 under a stream of nitrogen. The resulting masters were examined using a reflected light microscope to
129 confirm suitable patterning, and then incubated at 150°C for 3 min to hard bake the photoresist.. The
130 PDMS base (Sylgard™ 184) was mixed with its curing agent at a 10:1 ratio, after which air bubbles
131 were removed via centrifugation or under vacuum. The resulting formulation was poured onto the
132 masters to a depth of 5 mm, placed under vacuum again to eliminated bubbles, and then left overnight
133 at 70°C to allow full curing. The PDMS blocks were cut away from the masters to produce a stamp
134 with features corresponding to the microarchitecture of the master. Biopsy punches were used to
135 introduce cell culture medium reservoirs and hydrogel inlets, which can then be cleaned with residue
136 free tape to remove any obvious debris and rinsed in deionized water and isopropanol or ethanol before
137 drying in nitrogen. The stamps were placed in an air plasma system (HPT-200, Henniker Plasma) and
138 treated for one minute with the channels facing upward, alongside glass coverslips. The PDMS blocks
139 were then rapidly pushed firmly against the coverslip for 30 s to allow bonding and each chip was
140 carefully inspected to ensure the system was fully sealed. Finally, chips were autoclaved and left in an
141 oven for at least 48 h to allow the PDMS to recover its hydrophobicity.

142 **2.2 Cell Culture**

143 All primary cells used as part of this project were between passage numbers 3-6. Cells were cultured
144 in T75 Nunc™ EasYFlask™ cell culture flasks (ThermoFisher®, 156499) at 37°C and 5% CO_2 and
145 passaged at approximately 80% confluency. HUVECs were obtained from Lonza™ and cultured in
146 Endothelial Cell Growth Medium-2 (EGM-2, PromoCell® C-22111 or Lonza™ CC-3162). Human
147 primary pericytes were purchased from PromoCell® (PromoCell®, C-12980) and cultured in Pericyte
148 Growth Medium-2 (PGM2) also from PromoCell® (PromoCell®, C-28041). Detachment was
149 achieved using a pericyte detachment kit (PromoCell®, C-41200), requiring addition of 5 mL HEPES
150 buffered saline solution to the cells for washing, after medium aspiration, aspiration and addition of 5
151 mL Trypsin/EDTA 0.04%/0.03% for 3 min at room temperature. Trypsinisation was inhibited by
152 addition of 5 mL trypsin neutralization solution, after which cells were centrifuged and resuspended in
153 PGM2.

154 HCA2 fibroblasts - a human telomerase reverse transcriptase (hTERT) immortalized human dermal
155 fibroblast cell line (Stephens et al., 2004) were cultured in Dulbecco's modified Eagle medium
156 (DMEM, Gibco™) supplemented with 10% fetal bovine serum (FBS, Gibco™ 26140079), 1% L-
157 Glutamine and 1% penicillin-streptomycin (PS, Gibco™ 15140122). N/TERT cells (Hahn et al., 2000),
158 a human keratinocyte cell line featuring constitutive expression of human telomerase reverse
159 transcriptase (hTERT) were cultured in FAD medium (3:1 DMEM : Ham's F12 Medium
160 (ThermoFisher®, 11320074), supplemented with 1% PS, 1% L-Glutamine, 10% FBS, 0.5 µg/mL
161 human hydrocortisone (Fisher Scientific®, 35245-0010), 8.47ng/ml cholera toxin (Sigma-Aldrich®,
162 C8052-1mg), 10 ng/mL human epidermal growth factor (Peprotech®, AF-100-15) and 5 µg/mL human
163 insulin (Sigma-Aldrich®, 15500).

164 **2.3 Vasculogenesis Assays**

165 A bovine fibrin gel was used as a matrix for vascularization, following protocols reported in the
166 literature (Rohringer et al., 2014). In short, lyophilized bovine fibrinogen (Sigma-Aldrich®, F8630-

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167 25G) was dissolved in dPBS to a concentration of 20 mg/mL and filtered to ensure sterility. Separately,
168 a bovine thrombin solution of 4 U/mL was prepared by dissolving lyophilized bovine thrombin derived
169 from bovine plasma (Sigma-Aldrich, T4648-1KU) in 0.1% bovine serum albumin solution (BSA,
170 Sigma-Aldrich, A9418-50G) in dPBS, which was added to EGM-2 to achieve the final concentration.
171 Prior to injection into the device, 20 µL aliquots of fibrinogen solution were mixed with equal volumes
172 of thrombin solution in which was suspended appropriate densities of HUVECs, which triggered
173 formation of the final gel encapsulating endothelial cells.

174 For both chip designs, the wide central chamber was filled with 10 µL of a fibrin gel with a
175 concentration of 10 mg/mL, in which HUVECs were suspended at a density of 6-10 million cells/mL
176 by injection through the 3 mm central well with a cut 20 µL pipette tip. Both side channels were filled
177 with EGM-2 supplemented with 50 ng/mL Vascular Endothelial Growth Factor (VEGF) through filling
178 individual wells at one end of the chip and applying a negative pressure to the corresponding well for
179 each channel using a 1000 µL pipette, therefore allowing channel perfusion. Medium reservoirs were
180 filled equally. Chips were left to incubate at 37°C for approximately 45 min whilst HCA2 cells were
181 passaged and encapsulated in a collagen gel at a density of 2 million cells/mL. Collagen gels were
182 formed using rat-tail derived collagen I, as follows: working over ice, aqueous NaOH (1M) was added
183 to 10X dPBS (ThermoFisher®, 70011044). Rat tail derived type I collagen (Corning®, 354249) was
184 added to the buffer solution followed by HCA2 cells in dPBS and the mixture was well mixed, resulting
185 in a final collagen concentration of 5 mg/mL and a HCA2 density of 2 million cells/mL. 30 µL of
186 collagen gel solution containing fibroblasts was placed into the central well and allowed to set upon
187 the HUVECs-loaded fibrin gel cast at the bottom. Medium wells and the central well were filled with
188 EGM2 containing additional 50 ng/mL VEGF. Medium was replaced daily and after one week of
189 culture were fixed in 4% PFA in PBS and stained for imaging.

190 2.4 Organotypic Skin-on-a-Chip Formation and Vascularization

191 2.4.1 Dermal Equivalent preparation

192 Fibrin gels were prepared and injected into the vascular compartment of chips, either without cells, at
193 10^7 cells/mL HUVECs or 10^7 cells/mL HUVECs and 10^6 cells/mL pericytes. 20 µL was injected into
194 each chip. For vascularized chips, devices were filled with EGM-2 containing additional 50 ng/mL
195 VEGF, which was replaced daily and cultured for 4 days before addition of the dermal and epidermal
196 equivalents, to allow vascular development. Avascular chips were incubated with VEGF supplemented
197 EGM2 24 h before addition of the skin equivalent. For embedding of the dermal equivalent gel
198 precursor, solutions were stored on ice, using pre-cooled pipette tips kept in sterile sealed boxes at 0°C
199 and microcentrifuge tubes to avoid premature Matrigel ® setting. Medium was removed from the
200 reservoirs and central well of the devices. 250 µL high concentration (9 mg/mL) rat-tail derived
201 collagen I solution was injected into a microcentrifuge tube, followed by 100 µL Matrigel ®
202 (Corning®, 356234) and 50 µL 10X DMEM (ThermoFisher®, 11430030). After thorough mixing to
203 ensure homogeneity, the pH was adjusted to 7 by dropwise addition of sterile 1 M NaOH, in aliquots
204 of 5 µL, followed by further mixing, to a volume of approximately 405 µL. 50 µL sterile FBS was
205 added and mixed in, and the mixture was put aside on ice until primary fibroblasts had been passaged
206 and re-suspended to 1 M/mL in complete DMEM. 50 µL cell suspension was added to the
207 collagen/Matrigel ® mixture and mixed thoroughly to a final fibroblast density of 10^5 cells/mL. 20 µL
208 of the resulting cell suspension in gel precursor mixture was injected into the central well of the device
209 atop the fibrin scaffold. Injection was carried on the sides of the well, to avoid the formation of bubbles
210 allowing the matrix to rest evenly across the surface of the fibrin gel underneath and to fill the entire
211 diameter of the well.

212 #### 2.4.2 Epidermal Equivalent Preparation

213 Chips were incubated at 37°C for 1 h to allow gels to set, followed by passage of N/TERTs by addition
214 of 10 mL sterile versene solution for 5 min followed by addition of 2 mL sterile 0.05% trypsin-EDTA
215 for 5 min at 37°C. Cells were re-suspended to 10^6 cells/mL in FAD and 25 µL of the suspension was
216 injected on top of the gel down the side of the central well, to avoid bubble formation. After 10 min of
217 incubation at room temperature to allow N/TERT attachment to the top of the dermal equivalent, the
218 central well and medium wells were filled with FAD and chips were cultured overnight at 37°C and
219 5% CO_2. After approximately 18 h of incubation, medium was aspirated from the chips and fresh FAD
220 medium was replaced, which was done daily over the culture period. Chips were cultured for 7-14 days
221 at 37°C and 5% CO_2 before fixation in 4% PFA.

222 ### 2.5 Imaging

223 #### 2.5.1 On-Chip Immunological Staining

224 For samples that were imaged on-chip, fixation was carried out in 4% PFA in PBS for 15 min, followed
225 by washing in PBS thrice and incubated in 0.2% triton-X for 15 min. Primary and subsequently
226 secondary stains were applied in 3% BSA overnight followed by a PBS wash. Chips were loaded whole
227 into a sample holder mounted to the stage of the Zeiss™ LSM710 confocal microscope and imaged.

228 #### 2.5.2 Histology

229 ##### 2.5.2.1 Cryo-Histological Sectioning

230 For sectioning, immunostaining and confocal imaging, after fixation, samples were immersed in 30%
231 sucrose solution in PBS overnight by injection into lateral medium wells and the central well. This was
232 aspirated the following day and replaced with a 10% sucrose/ 7.5% porcine gelatin solution in PBS
233 and chips were left at 37°C for 15 min to allow the matrix to set. Simultaneously, some of the gelatin
234 solution was carefully pipetted into well plates avoiding bubble formation to a depth of approximately
235 1 cm, and the plates were left at 4°C for 15 min to allow the matrix to set. Tissues were harvested from
236 the central wells of the chips by slicing the coverslip from the PDMS carefully with a sharp scalpel and
237 cutting chunks from the remaining block until the organotypic was exposed on 3 sides. At this point,
238 the cultures were scooped from the PDMS and laid atop the set gels in well plates. More gelatin solution
239 was injected to immerse the cultures completely and the well plates were placed back at 4°C for 15
240 min to set. A cold hexane bath was made by dropping pieces of dry ice into hexane in a deep glass dish
241 with thick sides until it reached a temperature of between -30°C and -50°C. Orientation of the blocks
242 was assured by cutting the corners of the blocks to be attached to the sample holder of the cryostat.
243 Samples were frozen, sectioned and stored at -80°C until ready for staining.

244 ##### 2.5.2.2 Paraffin Embedded Section De-Waxing and Antigen Unmasking

245 Redundant human skin was obtained from healthy donors following plastic surgery procedures at the
246 Royal London Hospital or Phoenix Hospital, Essex. All donors provided written informed consent
247 according to local ethical approval (East London Research Ethics Committee, study number 2011-
248 000626-29 and East of England Research Ethics Committee, study number 21/EE/0057). Human skin
249 sections embedded in paraffin wax were dewaxed by incubation at 55°C for 10 min to melt the paraffin
250 followed by immersion in xylenes thrice, each for 15 min. To rehydrate the sections, slides were left
251 in a bath of ethanol and xylenes at a 1:1 ratio for 5 min, followed by sequential incubation in 100%,
252 95% and 70% ethanol in deionized water, and finally pure deionized water (all twice, each for 2 min).

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253 To unmask antigens hidden during sample fixation, the deionized water was aspirated and sections
254 were immersed in a 0.05% trypsin/0.1% $CaCl_2$ solution in deionized water, pre-warmed to 37°C, and
255 were incubated in a humid box for 20 min at 37°C. Samples were washed thrice more with dPBS before
256 staining.

257 **2.5.2.3 Section Staining & Slide Preparation**

258 Prior to staining, sections were allowed to equilibrate to room temperature for around 2 h. Sections
259 were outlined with a hydrophobic pen to facilitate staining and reduce volumes of required reagents.
260 4% PFA in PBS was dropped onto each section for 15 min, followed by a PBS wash and further 15
261 min incubation with 0.2% triton-X in PBS. Following another PBS wash, sections were blocked
262 overnight in 3% BSA solution in PBS at 4°C to reduce the risk of dehydration. Sections were incubated
263 with 15 µL primary antibody solution overnight in 3% BSA at 4°C, according to the stain required.
264 These included antibodies with the following antigens: keratin 14 (K14, AbCam® ab192055, 1:250),
265 Involucrin (ThermoFisher®, MA511803, 1:40), Ki67 (RM9106-50, 1:125), laminin-332 (AbCam®,
266 ab14509, 1:250), vimentin (AbCam®, ab8978, 1:150), TGM-1 (SigmaAldrich®, HPA040171, 1:150)
267 and CD31 (BioLegend®, 303126, 1:100). Secondary antibodies were also applied overnight in a 3%
268 BSA solution in PBS frequently alongside rhodamine-phalloidin and DAPI. These were Invitrogen®
269 antibodies purchased from ThermoFisher® attached to AlexaFluor™ fluorophores with emission
270 wavelengths of 488 nm or 647 nm. Secondary antibodies were raised against antigens according to the
271 animal origins of the primary antibodies and diluted 1:1000.

272 Sections were washed with PBS which was carefully aspirated, followed by addition of Fluoromount-
273 G™ Mounting Medium (ThermoFisher®, 00-4958-02) and a coverslip. Coverslips were allowed to
274 attach at room temperature overnight and slides were carefully cleaned with ethanol prior to imaging.
275 All fluorescent imaging was performed with the Zeiss™ LSM710 Confocal microscope.

276 **2.6 Image & Statistical Analysis**

277 For microvessel analysis, Z-stack files were loaded into Fiji and flattened to produce an image across
278 the depth of the microvascularised compartment. These were analyzed in AngioTool (Zudaire et al.,
279 2011) to find vessel junction number, overall vessel length and percentage area covered in any given
280 image by vasculature. To analyze sections, Z-Stacks were taken through the entire depth of sections
281 and loaded into Fiji. Individual slices were taken from the central image of each stack as representative
282 of that stack and used for data acquisition and figure production. Statistical analyses were carried out
283 using SPSS by IBM.

284

285 **3. Results**

286 **3.1 Vasculogenesis-on-a-Chip**

287 The proposed design of the microvascularised chip (Figure 1) is based on three channel chips
288 previously reported by Kamm, Jeong and others (Hernández Vera et al., 2008, Jeon et al., 2014). In
289 these microfluidic systems, three parallel linear channels are separated by micro-posts that can vary
290 significantly in size and shape from one report to another. This enables the central channel to be loaded
291 with an endothelial cell suspension in a gel precursor solution that is allowed to set *in situ* prior to
292 filling the side channels with tissue culture medium. The device initially selected featured a hexagonal
293 microfluidic chamber, 5 mm wide, with an integrated 3 mm diameter circular central well open to the

air, allowing a minimal gap of 1 mm between the circumference of the well and the edge of the chamber. Peripheral medium channels were separated from the central chamber by 2 parallel series of hexagonal posts, 380 µm long and 90 µm wide, either side of the microfluidic culture compartment. The morphology and homogeneity of vascular networks formed in such systems were first investigated.

Vascularization was achieved via a vasculogenesis protocol adapted from the literature (Kim et al., 2016), consisting in de novo capillary formation of HUVECs encapsulated within the fibrin gel in the central compartment. The impact of cell density on vascular network formation was first examined (Supplementary Figure S1). HUVECs were introduced in the central gel compartment at 6, 8 and 10 million cells/mL. After 7 days of culture, cells formed interconnected vascular networks that were luminated, as evidenced by confocal microscopy (Figure 2). Cell densities had no significant impact on the morphology of the networks formed, within the range tested (Supplementary Figure S1). The total cross-section area of networks, the total length of capillaries and their degree of branching were comparable. However, we noted that the quality of the networks differed significantly depending on their position within the channel. Close to the central well (position A), vascular networks were denser (higher cross-section areas, length and branching densities) than in more distant areas, close to the injection channels (position B, Figure 2D-F).

To encourage more uniform vascularization, the device was redesigned. The hexagonal microfluidic culture compartment flanked by straight parallel peripheral medium channels was replaced with a round compartment with circumferential 1 mm wide channel (Figure 2B). Hexagonal posts were redesigned to follow the circumference of the central channel. Given the lack of impact of HUVEC density on vascularization, within the range tested, cells were seeded within these redesigned chips at a density of 8 million cell/mL (suspended in 10 mg/mL fibrin gel).

Vascularization surrounding the central channel of round chips was comparable to hexagonal chips (Figure 2C-F), without significant changes in vascular network coverage, density or branching. In contrast to what had been observed with hexagonal chips, comparison of images taken near the central well of round chips with those taken near injection channels revealed no significant difference in network architecture either (Figure 2D-F). Levels of coverage by vascular networks were comparable near the central well and injection channels, and associated vascular networks were as dense and branched. This suggests that improved medium diffusion in different compartment within round chips enables improved formation of homogenous vascular networks. Examination of confocal z-stacks taken of corresponding vascular compartments also indicated the formation of a lumenated structures (20 µm of internal diameter) in networks formed in round chips (Figure 2G-H). Therefore our data indicate that round chips support the growth of a mature and more homogenous microvascular networks within multi-compartment microfluidic chips. This model was therefore adopted for the development vascularized skin-on-a-chip systems.

3.2 Establishment of a Skin-on-a-Chip Model

To engineer the full thickness dermal equivalent within our microfluidic model, we proposed to introduce dermal fibroblasts within the central well of round chips. As a first step, we introduced the dermal compartment on top of an acellular fibrin gel, which could subsequently be vascularized, as presented in Figures 1 and 2. A collagen I/Matrigel dermal equivalent matrix was selected, based on protocols established for transwell skin equivalent culture (Enjalbert et al., 2020). Fibrin was injected into the microfluidic compartment of the chip and devices were incubated at 37°C for 5 min, followed by perfusion of medium channels and filling of all on-chip wells with complete DMEM. Chips were left for 24 h at 37°C to allow bubbles forming at the medium-hydrogel interface to disperse. The

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338 medium was then removed from chips and 20 µL of dermal equivalent matrix (rat-tail derived collagen
339 I and Matrigel with pH adjusted to 7) together with 10^5 cells/mL primary human dermal fibroblasts
340 were injected in the central well and allowed to set at the surface of the fibrin compartment, for 1 h at
341 37°C. N/TERT keratinocytes were passaged and resuspended at 1 million cells/mL in FAD medium,
342 and 25 µL of the suspension was injected on top of the dermal equivalent. Devices were incubated for
343 10 min at room temperature to facilitate initial N/TERT attachment, followed by the filling of medium
344 wells with FAD medium. After 24 h incubation, the medium was removed from the chip and replaced.
345 To examine the impact of air-liquid interface culture, which is typically used for the formation of skin
346 equivalents to encourage epidermal stratification and differentiation, dermal equivalents on chips were
347 either left submerged (fresh FAD introduced into the central well) or exposed to air. Culture was
348 continued for a total of 7 or 14 d, followed by fixation and embedding for histology and immunological
349 staining.

350 At day 7, keratinocytes formed confluent epidermal layers across the surfaces of dermal matrices and
351 positive for basal K14 (Supplementary Figure S2). After 14 days of culture, both in submerged
352 conditions and at the air-liquid interface, we observed the formation of confluent stratified epidermal
353 layers, atop the dermal equivalent matrix (Supplementary Figure S2). Infrequent proliferative cells
354 (Ki67 positive) were observed in the basal epidermal layers of epidermal equivalents cultured
355 submerged in medium (Figure 3). Although this localization and such overall low rates are typical of
356 skin equivalents and proliferation rates observed in vivo (Heenen et al., 1998, Commandeur et al.,
357 2012), the lack of Ki67 positive cells in tissues formed at the air-liquid interfaces was surprising. In
358 agreement with such observations, we also observed significantly thicker epidermal equivalents in
359 submerged cultures (46 +/- 16 µm) compared to those formed at the air-liquid interface (24 +/- 12 µm,
360 Figure 3C). In addition, the abundance of the basement membrane protein laminin-332 was
361 significantly higher in submerged cultures (by a factor of >2 fold, Figure 3C), suggesting that
362 keratinocytes in these cultures were also more active at remodeling corresponding interfaces and
363 recreating a normal functional basal lamina. The thickness of the basal marker keratin 14 was also
364 significantly increased in submerged cultures (28 +/- 10 µm) compared to those kept at air-liquid
365 interfaces (15 +/- 10 µm, Figure 4). Finally, whilst expression of the terminal differentiation marker
366 involucrin was observed in the upper-epidermal layers of both cultures, the thickness of the associated
367 differentiated layer was significantly increased in submerged cultures (3.5 +/- 2.2 µm) compared to
368 those kept at the air-liquid interface (0.5 +/- 0.2 µm, Figure 4). However, relatively weak expression
369 of the dermal fibroblast marker vimentin was observed in the dermal compartment of both cultures,
370 and very weak staining for the epidermal differentiation marker transglutaminase 1 (TGM-1) was
371 observed in the stratified layers of corresponding epidermal compartments (Figure 4B).

372 **3.3 Microvascularised Skin-on-a-Chip Model**

373 The microvascularised model developed in a fibrin matrix, and the stratified human skin equivalent
374 were then combined in round chips. In addition, owing to their impact on the maturation and
375 stabilization of microvasculatures (Campisi et al., 2018, Hirschi and D'Amore, 1996, Kim et al., 2013),
376 we investigated the impact of pericytes on the resulting vascularized skin equivalents. HUVECs (10^7
377 cells/mL) were seeded alone or together with human primary pericytes (10^6 cells/mL) in a 10 mg/mL
378 fibrin within the central channel of round chips. Side channels and the central well were supplemented
379 with EGM-2 medium containing 50 ng/mL. After 4 days of culture to allow establishment of a mature
380 microvasculature, the dermal equivalent was introduced into the central well, followed by seeding of
381 N/TERT keratinocytes after 1 h, as previously described. Medium channels and the central well were
382 filled with FAD medium and culture was continued for 14 d, under submerged conditions
383 (Supplementary Figure S3). Cultures were fixed and dissected from chips for histological processing

384 and immunological staining. Sections taken from our samples were compared with *ex vivo* human skin
385 sections.

386 Resulting tissues displayed a well-structured multi-layer epidermal equivalent with flattened stratified
387 squamous cells (Figure 5 and Supplementary Figures S4 and S5). Although the overall thickness and
388 level of flattening and stratification of the epidermal compartment remained lower than what can be
389 observed in sections from human skin, epidermal equivalents were more developed in the presence of
390 microvasculatures (whether with or without pericytes). Rete ridges present in *in vivo* human skin were
391 also not observed in our HSE-chip models, although these are not generally recapitulated in other
392 human skin equivalent reported (Choudhury and Das, 2021). The overall depth of the epidermal
393 compartment significantly increased in vascularized chips (86 +/- 20.3 µm, for vascularized chips in
394 the presence of pericytes, compared to 64 +/- 26.5 µm, for avascular chips; Figure 5C). Although these
395 thicknesses are in line with those reported for skin equivalents (Chopra et al., 2015, Wei et al., 2017),
396 our results indicate that vascularization impacts on the establishment of the epidermal structure in skin
397 equivalents.

398 To study this phenomenon further, we investigated stainings of tissue sections. The impact of
399 vascularization on the basal compartment of the epidermal equivalent was relatively modest. We
400 observed a small increase in the thickness of the basement membrane constituent laminin 332 in
401 vascularized chips, compared to avascular models and *ex vivo* skin sections (Figures 5B and E). This
402 increase was not statistically significant when comparing avascular and vascularized chips, but P values
403 decreased to <0.001 when pericyte/HUVEC vascular networks were used in the vascularised
404 compartment, compared to human skin sections. This modest change in basement membrane did not
405 translate into differences in expression of the basal marker keratin 14 (Figure 6). Similarly, Ki67
406 positive cells in the epidermis were confined to the basal layer in all conditions and their frequency
407 was comparable (Figure 5D). However, these observations contrasted with the lack of Ki67 expression
408 in the basal compartment of *ex vivo* human skin sections, in line with previous observations made on
409 comparable human sections (Heenen et al., 1998).

410 Epidermal differentiation marker expression was more established and consistent with the addition of
411 a vascularized dermal compartment: the thickness of involucrin positive layers in chips containing
412 either HUVECs alone or alongside pericytes (8 +/- 5.8 and 8 +/- 3.8 µm, respectively) compared to
413 avascular models (3 +/- 2.2 µm, Figures A and D). However, involucrin expression remained weaker
414 than in human skin sections (10 +/- 2.9 µm). Similarly, staining for transglutaminase was significantly
415 more pronounced in vascularized, compared to avascular chips. Beyond expression levels, the
416 localization of transglutaminase was also more obviously localized at the upper layers of the epidermis
417 in vascularized HSE-chips, recapitulating well the pattern of expression typical of human skin sections
418 (Figure 6B). We did not observe a significant impact of vascularization on the thickness of
419 transglutaminase expression (Figure 6E), but expression was not confined to the uppermost layers of
420 the epidermal equivalents in avascular chips and could be seen in layers directly above the basal layer.

421 The impact of vascularization on the dermal compartment could also be clearly observed. CD31
422 positive structures were obviously only observed in vascularized models, as would be expected from
423 the presence of endothelial cells (Figure 5B). Associated with such structures, we clearly observed an
424 increase in vimentin positive cells in vascularized chips, compared to avascular cultures (Figure 6B).
425 Vimentin positive cells were clearly denser in human skin histological sections, but this reflects a
426 general higher degree of cellularity in normal tissue dermis, compared to the in vitro models
427 established.

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428 Further characterization of the vascular compartment was carried out next. We observed extensive
429 dermal microvascular networks spanning the microfluidic compartments underlying the central well
430 cultures of chips loaded with HUVECs alone (bright field images, Figure 7), confirmed via staining
431 for the endothelial marker CD31 (Figure 7D). In the presence of pericytes, vascular networks were also
432 clearly observed, but displayed altered morphologies, in agreement with similar systems reported in
433 the literature (Kim et al., 2015b). Pericyte co-cultures displayed reduced total length and branching
434 (Figures 7A-B). Such phenotype is proposed to be associated with the maturing and stabilizing role of
435 pericytes, enabling to better retain the stability of resulting networks. Mechanisms involved in this
436 behavior remain partially unclear, but may involve cytokine secretion (e.g. angiopoietin-1, angiogenin,
437 HGF, TGF-a and TNF) and perivascular matrix remodeling (Newman et al., 2011). Whilst others
438 reported an impact on endothelial cell-cell junctions (Jeon et al., 2014, Jeon et al., 2015), our previous
439 results did not confirm such effect.

440 To investigate the architecture of the microvasculatures integrated into the dermal equivalents in our
441 cultures, we examined the formation of capillaries in the sections generated (Figure 7D). CD31 and
442 laminin stainings clearly established the formation of rosettes associated with the formation of
443 lumenated capillaries (Figure 7D), in agreement with the recruitment of laminin at the basal membrane
444 of micro-capillaries (Black et al., 1998, Schurr et al., 1999, Kubota et al., 1988). The density of these
445 structures was clearly increased in HUVEC-pericytes co-cultures, indicating a better retention of the
446 vascular networks within these models (Figures 7C and D). The average external diameter of capillaries
447 determined from sections, although approximative as only relying on the ability to identify clear
448 transverse sections, were 12 +/- 6 µm for monocultures and 13 +/-6 µm for HUVEC/pericytes co-
449 cultures, which correspond to the external diameter of dermal arterial capillaries *in vivo*, identified
450 from human skin sections (Braverman, 2000). Overall, our results are evidencing the supportive role
451 played by pericytes in our model, maintaining the microvasculature integrity despite the use of mixed
452 culture media in our protocol (Campisi et al., 2018, Hirschi and D'Amore, 1996).

453 **4. Discussion**

454 Altogether these data demonstrate that our chip design enables the formation of a microvascular
455 network embedded in a multi-compartment microfluidic system and underlying the formation of a
456 dermal/epidermal skin. The design of a round central chamber and circumferential channels enables
457 the formation of a more homogenous microvasculature within the central compartment (Figure 2), in
458 agreement with previous data presented for a vascularized ocular model (Ko et al., 2019b). It is also
459 expected that the circular design selected will result in more homogenous cytokine and growth factor
460 gradients within the different compartments, as was previously described in a model of pluripotent
461 stem cell differentiation (Manfrin et al., 2019) and as briefly discussed in a model of central nervous
462 system axonal growth, across the diameter of a microfluidic compartment (Park et al., 2009).

463 A surprising result from our study is that skin equivalents, in the absence of microvasculature, form
464 more mature stratified epidermal compartments in submerged conditions, compared to those cultured
465 at the air-liquid interface. This contrasts with the literature indicating that culture at the air interface,
466 for skin equivalents grown in transwell systems, allows improved stratification and accurate epidermal
467 differentiation marker expression (Prunieras et al., 1983, Rosdy and Clauss, 1990). These results may
468 be explained by the smaller dimensions of the constructs generated and the lower volumes of media
469 required for their culture. Indeed, the smaller dimension may result in higher local oxygen
470 concentrations, impacting on keratinocyte commitment and stratification (Ngo et al., 2007). In
471 addition, the smaller volumes may allow greater accumulation of cytokines and growth factors
472 promoting stratification. It may also be that, the lower volumes of media used in culture at air-liquid

interfaces in our chips result in increased cell death, and that the added volume associated with submerged culture helps limiting such process, as demonstrated by Lee et al. in 2017 in their vascularized skin-on-a-chip (Lee et al., 2017). Indeed, tissues formed at the air-liquid interface displayed significantly reduced Ki67 expression, compared to submerged systems (Figure 3).

Culture on microvascularized bed further improved the stratification and differentiation of skin equivalents. The use of pericytes was found to have weak but statistically significant impacts on the density of capillaries and their apparent lumenation in our skin equivalents (Figure 7). Therefore, beyond their initial impact on the morphology of vascular networks, pericytes may contribute to the complementarity of vascularized models with other tissue equivalents, allowing the formation of more complex vascularized tissue models. This may be associated with perivascular matrix remodeling (Avolio et al., 2017, Bergers and Song, 2005) and cytokine secretion, which were found to promote the stability of vascular networks (Hurtado-Alvarado et al., 2014, Sounni et al., 2011).

Improved epidermal stratification and differentiation upon addition of a microvascular component has previously been demonstrated in both *in vivo* mouse and *in vitro* systems (Baltazar et al., 2020). Conversely, Rochon et al. have shown that various epithelial cell types have a direct impact on endothelial vessel physiology *in vitro* (Rochon et al., 2009). This is likely due to increased bi-directional diffusion of secreted growth factors and cytokines in the surrounding microenvironment, known to impact differentiation in keratinocyte populations (Ansel et al., 1990, Gröne, 2002). Stromal fibroblasts in the dermal equivalent matrix also likely contribute to the secreted factors to encourage improved epidermal stratification; this has been demonstrated previously in human *in vitro* systems, which commonly report an undifferentiated, unstratified epidermis' with a reduced thickness and poor localization of upper-epidermal and basement membrane markers in the absence of proximal fibroblasts (Saintigny et al., 1993). In 2020, Russo et al. published an extensive review on this topic (Russo et al., 2020). Pericytes alongside HUVECs have been shown to increase epidermal thickness and the expression of cytokeratins 1/10 and laminin 332 in *in vitro* skin models, which was also confirmed in engraftment experiments into a murine setting for a duration of 4 weeks (Baltazar et al., 2020). In agreement with this literature, stromal cell interactions are proposed to improve epidermal architecture in our model.

Overall, our results demonstrate the feasibility of hierarchically structured vascularized-skin-on-chip equivalents, paving the way for the embedding of these systems for testing of therapeutics safety and efficacy testing in a human context in vitro. Such models will allow testing of systemic as well as topical delivery in realistic scenarios, in platforms that may be compatible with high throughput testing. This still requires the engineering of multi-chip plate systems compatible with high throughput formats, in analogy to some of the epithelial/endothelial tissue models developed on the Emulate platforms (Si et al., 2021), and the parallelization of multi-cellular culture and embedding in the resulting micro-engineered chips. This latter process may require particular attention from the research community as parallelized and automated multi-cell culture systems have not been engineered to date.

3 Conflict of Interest

The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

4 Author Contributions

JEG and JC: conceptualization and project administration. CFEJ and SC: data curation, formal analysis, and validation, visualization and writing – original draft. JEG and JC: funding acquisition, resources

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and supervision. SC: data curation. JEG: writing – review and editing, writing – original draft. All authors contributed to the article and approved the submitted version.

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7 Supplementary Material

Supplementary immunostainings of vascularised chips and histology sections can be downloaded from XXXX and are available from the authors.

8 Data Availability Statement

The raw data supporting the conclusions of this article will be made available by the authors, without undue reservation.

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