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A Biofabricated Vascularized Skin Model of Atopic Dermatitis for Preclinical Studies

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Abstract

Three-dimensional (3D) biofabrication techniques enable the production of multicellular tissue models as assay platforms for drug screening. The increased cellular and physiological complexity in these 3D tissue models should recapitulate the relevant biological environment found in the body. Here we describe the use of 3D bioprinting techniques to fabricate skin equivalent tissues of varying physiological complexity, including human epidermis, non-vascularized and vascularized full-thickness skin tissue equivalents, in a multi-well platform to enable drug screening. Human keratinocytes, fibroblasts, and pericytes, and induced pluripotent stem cell (iPSC)-derived endothelial cells were used in the biofabrication process to produce the varying complexity. The skin equivalents exhibit the correct structural markers of dermis and epidermis stratification, with physiological functions of the skin barrier. The robustness, versatility and reproducibility of the biofabrication techniques are further highlighted by the generation of atopic dermatitis (AD)-disease like tissues. These AD models demonstrate several clinical hallmarks of the disease, including: (i) spongiosis and hyperplasia; (ii) early and terminal expression of differentiation proteins; and (iii) increases in levels of pro-inflammatory cytokines. We show the pre-clinical relevance of the biofabricated AD tissue models to correct disease phenotype by testing the effects of dexamethasone, an anti-inflammatory corticosteroid, and three Janus Kinase inhibitors from clinical trials for AD. This study demonstrates the development of a versatile and reproducible bioprinting approach to create human skin equivalents with a range of cellular complexity for disease modelling. In addition, we establish several assay readouts that are quantifiable, robust, AD relevant, and can be scaled up for compound screening. The results show that the cellular complexity of the tissues develops a more physiologically relevant AD disease model. Thus, the skin models in this study offer an *in vitro* approach for the rapid understanding of pathological mechanisms, and testing for efficacy of action and toxic effects of drugs.

Keywords: 3D bioprinting, atopic dermatitis, *in vitro* model, skin, vascularization, preclinical study

1. Introduction

Biofabricated three dimensional (3D) tissues that recapitulate the morphology and physiology of native human tissues are being developed for regenerative medicine, disease modelling and drug screening applications. 3D bioprinting is an

emerging tissue engineering technology that enables spatially controlled biofabrication of 3D tissues with varying degrees of cellular and physiological complexity [1,2]. The presence of multiple cell types in the tissues mimic more faithfully cell-cell interaction and crosstalk that occurs in native tissues compared to single cell type organotypic constructs[3,4].

Building such physiologically-complex cellular models with high human fidelity and reproducibility relies on technical aspects such as consistent and scalable sources of relevant cells, extracellular matrix (ECM) components for bioinks, and bioprinting techniques with high resolution[5]. The reproducible biofabrication of native-like tissues in a screenable format should enable the development of pre-clinical assay platforms. These assay platforms can be used to investigate basic biology and the underlying cellular and disease mechanisms, in a physiologically and pathologically relevant microenvironment, leading to better predictions of the effects of drugs in humans. Although the development and use of biofabricated tissue models for pre-clinical studies is increasing in popularity, there is still a need for experimental outcomes to explore what degree of physiological complexity is needed to demonstrate how accurate these models are in predicting clinical drug responses. Complex tissue features such as vascularization, innervation, or immune components might be critical to generate a disease relevant model but they remain very challenging to integrate into biofabricated tissues.

Skin is the largest organ of human body and it is the first line of protection from external microorganisms and other biological and physical insults [6]. Animal models have been extensively used to study human skin physiology and pathology, and for drug discovery. However, animal models often poorly represent and predict drug responses in humans because of the species differences [7]. Alternatively, human *ex vivo* skin tissues have been used for testing dermal toxic effects of chemicals. However, there are limited sources of skin explants, especially considering the variations due to ages, body sites and genders of the samples collected. Thus, obtaining enough samples to do large scale drug testing is often challenging[4]. Commercially available *in vitro* skin tissue models of human epidermis or full-thickness skin with dermis and epidermis layers generated using human primary dermal fibroblasts and keratinocytes are being used for toxicity risk assessment of chemicals [8–10]. However, these skin models are missing important physiological features, such as vasculature, which are critical for most disease modelling. For skin tissues, the dermal vascular endothelial cells (EC) are a critical component during initiation and progression of inflammatory skin diseases [11,12]. It has also been previously reported that vascularization of engineered skin tissues improves nutrient and oxygen delivery for long term tissue viability with the goal of enhancing physiological relevance [3,13–20]. Recent efforts in the development of vascularized tissues are mostly using organ-on-a-chip approaches[21,22]. These systems enable perfusion of active fluid flow through the premade channels covered by vascular EC monolayer, and chemical compounds can be delivered through the channels. However, these are low throughput systems which require sophisticated manipulation during the fabrication process and testing. A limited number of samples

can be tested thus preventing its practical application as pre-clinical development platform.

Here we describe a robust, versatile and reproducible 3D bioprinted approach to produce skin tissue models of increased cellular complexity, in a multi-well plate format. Skin equivalents with different cellular complexity, including reconstructed human epidermis (RhE), non-vascularized full-thickness skin (FTS), and vascularized full-thickness skin (VFTS) were produced using the biofabrication protocols developed. The skin tissue biofabrication protocols described here use a commercially available bioprinter and consumables to enable widespread use in the community. The main novelties of this skin biofabrication protocol include: 1) a bioink that includes neonatal fibroblasts, pericytes, and human induced pluripotent stem cell (iPSC)-derived endothelial cells with a fibrin-based hydrogel to make a vascularized dermis; 2) bioprinting the above bioink in a defined spatial pattern on the basal side of an electrospun poly(lactic-co-glycolic acid) (PLGA) biodegradable scaffold in a transwell format to generate a vascularized dermis. The bioink is bioprinted in a defined spatial pattern that allows quantitation of vasculogenesis and angiogenesis using fluorescence cellular microscopy. This bioprinting skin tissue protocol provides skin equivalents with multiple cell types in the dermis, including self-assembled blood vessels, for increased physiological relevance compared to traditional skin equivalents, which only have fibroblasts.

The biofabricated skin tissues developed were used to generate disease models of Atopic Dermatitis (AD). AD is a complex inflammatory skin disease that involves multiple cell types [23]. During the onset and progression, allergen-stimulated T helper type 2 (Th2) cells drive an immune reaction that triggers inflammatory signalling pathways through secreted pro-inflammatory cytokines. The positive feedback of signalling pathways leads to overproduction of inflammatory cytokines in chronic AD. Th2 related cytokines interleukin 4 (IL-4) and interleukin 13 (IL-13) are well known to be involved in AD pathophysiology, inducing the principal signalling cascades through Janus kinase/signal transducer and activator of transcription protein (JAK-STAT) pathway [24]. Many ongoing clinical studies include testing of generic anti-inflammatories and inhibition of the JAK-STAT pathway as a therapeutic target to intervene in AD progression and to rescue the compromised skin barrier function [25,26]. The AD-like skin tissue models demonstrated several clinical hallmarks of the disease, including: (i) spongiosis and hyperplasia; (ii) early and terminal expression of differentiation proteins; and (iii) increases in levels of pro-inflammatory cytokines. We also demonstrate the pharmacological validity of the skin tissue model for drug screening by showing how JAK inhibitors in clinical development for AD can rescue disease phenotypes, including tissue integrity and molecular outcomes.

2. Material and Method

2.1 Cell Culture

Neonatal human dermal fibroblasts (NDFs) were purchased from ATCC (ATCC, PCS-201-010, Manassas, VA, USA). Human iPSC endothelial cells were purchased from FUJIFILM (FUJIFILM Cellular Dynamics, R1022, WI, USA) and maintained in iCell endothelial cells medium (Supplemental table 1). Neonatal human epidermal keratinocytes (NHEKs) were purchased from Sciencell (Sciencell, 2100, Carlsbad, CA, USA). NDFs were maintained in MEM medium supplemented with 5% FBS (Thermo Fisher Scientific, Waltham, MA, USA). NHEKs were maintained in keratinocyte growth medium (KGM) purchased from Sciencell (Sciencell, Carlsbad, CA, USA). Human placental microvascular pericytes were purchased from agio-protemomie (Angio-proteomie, Boston, MA, USA) and cultured in MEM medium supplemented with 5 % FBS (Thermo Fisher Scientific, Waltham, MA, USA). All the cells were maintained at 37 °C under a 5% CO₂ atmosphere. Cells of passage number 3-4 were used. All the cultures were routinely monitored for mycoplasma contamination using MycoAlert PLUS mycoplasma detection kit (Lonza, Walkersville, MD, USA).

2.2 Biofabrication of reconstructed human epidermis (RhE)

RhE was generated adapting previously described protocols [27]. In brief, polycarbonate HTS-96 transwell with 0.4 µm pore size (HTS Transwell-96, 3381, Corning, NY, USA) were coated with 50 µL rat tail collagen (100 µg/mL, Corning, NY, USA) in phosphate buffered saline (PBS, Corning, NY, USA), for 1 hour at room temperature. The transwell insert was then rinsed once with 100 µL of sterile cold PBS. Fifty µL of a human neonatal primary keratinocytes cell suspension (2 x 10⁵ cell/cm² in KGM media) were seeded by pipetting on top of the membrane of the transwell insert. The cells were cultured submerged in 50 µL KGM media for 2 days. Afterwards, the media was changed to keratinocyte differentiation media for another 24 hours. The keratinocyte differentiation media consists of CnT-PR-3D media (Zen-Bio, Research Triangle Park, NC, USA) and Dulbecco's Modified Eagle Medium (DMEM, Thermo Fisher Scientific) with a 3:2 ratio. The tissues were then cultured in an air-liquid interface (ALI) with 400 µL of keratinocyte differentiation medium in the base plate well, with no media in the transwell, for 8 days. The transwell insert was placed on a custom lifter (6 mm height), between the base plate and the insert tray, in order to add enough volume of media in the base plate well. The culture medium was changed every other day.

2.3 A Customized transwell insert for bioprinting

The HTS 24 well transwell (Falcon 351185, Corning, NY, US) membrane was replaced with an electrospun biodegradable scaffold. The scaffold was made of poly(lactic-co-glycolic acid) (PLGA) (50:50, 1.0 inherent viscosity) nanofibers with 900 nm diameter and 4 µm thickness (BioSurfaces, Ashland, MA) to achieve better cell adherence and migration. Its half degradation time was about 6 weeks. Plasma treatment was applied to activate the cell adherent surface and sterilization purposes. A customized biocompatible O-ring (Ultimaker B.V, Netherlands) was glued onto the bottom of the transwell insert using bio-compatible adhesive (Kwik-cast sealant, world precision instrument, Sarasota, FL, USA). The polycarbrolactone O-ring was used to prevent any leakage from the bioprinted hydrogel. The transwell inserts were then treated with 200 µL fibronectin dissolved in deionized water (0.03 mg/mL, Thermo Fisher Scientific, Waltham, MA, USA) for 1 hr at room temperature, followed by aspirating and air-drying overnight. The basal side of the assembled transwell scaffold insert was treated with oxygen plasma for 30 minutes before bioprinting. The final culture area was 0.33 cm² for the 24 well HTS plate..

2.4 Bioprinting of a 3D human vascularized dermal equivalent

The schematics of the bioprinting of the full thickness skin tissues are shown in Figure 1(a). A mixture of fibrinogen (2.5 mg/mL, Sigma-Aldrich, St. Louis, MO, USA), Novogel component 2, a thermo-responsive hydrogel, (60 mg/mL, Organovo, Sand Diego, CA, USA), and aprotinin (0.075 U/mL, Sigma-Aldrich, St. Louis, MO, USA) in dPBS was prepared as bioprinting hydrogel. The bioprinting hydrogel was kept in a 37°C water bath for 1 hour before use. 8 x 10⁶ neonatal fibroblasts, 7x10⁶ iPSC endothelial cells, and 0.7x10⁶ pericytes were suspended in 1 mL of the hydrogel in a 2.5 mL syringe (RegenHU, Switzerland). As Novogel component 2 determined bioink gelation, the bioink was kept in the fridge for 10 minutes to allow gelation before assembling on to the bioprinter. The predefined construct was biofabricated using RegenHU 3D Discovery™ Evolution bioprinter with an extrusion-based printing head. The needle with a 250 µm diameter was used. Four layers of stripes with 10 µL bioink was printed in a zig-zag pattern onto the bottom of the 24 well HTS transwell insert, which was subsequently overlaid with 70 µL of a mixture of fibrinogen (4.5 mg/mL), aprotinin (0.075 U/mL) and thrombin (1 U/mL), in dPBS dispensed by pipetting. Fully supplemented dermal medium, FDM-A (Supplementary Table 1) with 1U/mL thrombin, was added after 15 minutes, and for 24 hrs, to allow partial fibrinogen polymerization. The tissue was kept at room temperature for 2 hrs for further polymerization and moved to the incubator subsequently. FDM-A without thrombin was used after the first 24 hrs. The dermal layer was incubated for 7 days in a

submerged condition. The medium was changed every other day.

2.5 Generation of full-thickness skin equivalent (FTS) and vascularized full-thickness skin equivalents (VFTS)

On day 7 after bioprinting the dermis equivalents (either vascularized or non-vascularized), a keratinocyte cell suspension ($2.0 \times 10^5 / \text{cm}^2$) were pipetted onto the apical side of the transwell insert while the bioprinted dermis remained on the bottom side. The keratinocytes were suspended in 100 μL of serum-free KGM medium. The tissue was incubated with 200 μL KGM medium in the apical side and with 800 μL FDM-A medium in the base transwell compartment. A customized fitted lifter was added to the transwell in order to create enough medium volume in the base compartment. After 48 hrs, the apical medium was changed to differentiation medium and incubated for another 24 hrs. After 3 days, the tissue was finally cultured at ALI for 7 days with 800 μL FDM-B in the base transwell compartment. The tissue was cultured with FDM-C from ALI day 8 to the day of harvest.

2.6 Bioprinting of 3D human dermal equivalent

The whole procedure of generating FTS is exactly the same as VFTS except, instead of having three type of cells in the bioink, there are only fibroblasts (ATCC, PCS-201-010, Manassas, VA, USA).

2.7 Atopic dermatitis-like skin equivalent generation

From ALI day 1, T_H2 cytokine IL-4 (20 ng/mL, PeproTech EC Ltd.) was added to the culture medium to generate AD-like FTS and VFTS. To test the effect of anti-AD compounds, three JAK inhibitors were selected. 20 μM tofacitinib, baricitinib, or ruxolitinib (from DMSO stocks) was added to the medium. JAK inhibitors were added simultaneously with IL-4. Both IL-4 and JAK inhibitors were refreshed every other day for 10 days of ALI period. The final concentration of DMSO was 0.1 % in the system. Both normal and AD control tissues were supplemented with 0.1 % DMSO. Dexamethasone was chosen to prove the reaction of targeting T-cells instead of JAK/STAT6 pathway.

2.8 Transepidermal electrical resistance (TEER) assay

TEER measurements are acquired every other day from air liquid interface day 6 using automated TEER measurement system (world precision instrument, Sarasota, FL, USA). The transwell was filled with 300 μL dPBS in the apical region. The contribution of the scaffold with O-ring was measured and subtracted from the sample values. TEER final values in $\Omega \cdot \text{cm}^2$ are obtained by multiplying the electrical resistance

with skin surface area. Any tissue with a TEER value lower than $500 \Omega \cdot \text{cm}^2$ was not used in this research [28].

2.9 Cytokine measurements

Cytokines were measured using plate-based assay from Meso Scale Discovery (MSD) (MesoScale Discovery, Gaithersburg, MD, USA). V-PLEX custom human biomarkers (Proinflammatory Panel 1, Cytokine Panel 1, Chemokine Panel 1, Human Angiogenesis 1) were purchased from MSD (K151A9H-1). All reagents were provided with the MSD kits. The standards were reconstituted in the assay diluent provided. Assay diluent (25 μL) was added to all wells and the plate sealed and incubated for 1 hour at room temperature on an orbital shaker (750 rpm). Samples, standards and controls were added at 25 μL per well. The plate was sealed and incubated for 2 hours at room temperature on an orbital shaker (750 rpm). At the end of the incubation the wells were washed three times using 150 μL PBS + 0.05 % Tween 20 (Sigma-Aldrich, St. Louis, MO, USA). Detection antibody was added at 25 μL per well, and the plate sealed and incubated for 1 hour at room temperature on an orbital shaker (750 rpm). At the end of the incubation the plate was washed three times as before. 150 μL of the MSD Read Buffer was added to each well and the MSD plates were measured on MESO QuickPlex SQ 120. The raw data was measured as electrochemiluminescence signal (light) detected by photodetectors and analyzed using the Discovery Workbench 3.0 software (MSD) (N=3).

2.10 Whole tissue fixation and immunostaining

The reconstructed skin tissues were fixed in 4 % vol /vol paraformaldehyde overnight at 4 °C, followed by three 10 minute washes in PBS. Tissues were then cut out using biopsy punches from the transwell insert. All subsequent steps were performed at room temperature on a VWR DS-500 shaker plate at 90 rpm. Tissues were permeabilized and nonspecific staining was blocked by incubation in 0.1 % Triton X-100, 2 % normal goat serum, 1 % bovine serum albumin (BSA) in PBS for 1 hour. Tissues were incubated in primary antibody diluted in 1 % BSA solution overnight (Supplementary, Table 2). Next, tissues underwent three 10 minute washes in PBS. Tissues were then incubated in secondary antibody with diluted 1 $\mu\text{g/mL}$ 4',6-diamidino-2-phenylindole (DAPI) in 1 % BSA solution for 4 hours (Supplementary, Table 1). Tissues are then returned to PBS washes three times for 10 minutes each.

2.11 Tissue embedding and cryosectioning

The biofabricated human vascularized tissues were fixed and washed as above, then taken through a gradient of 15 % and 30 % wt/vol sucrose in PBS over 6 hours. Whole tissues were removed from sucrose, blotted dry, and embedded in

Tissue-Tek CRYO-OCT Compound (Andwin Scientific, Tryon, NC, USA). Blocks were stored at -80 °C, sectioned on a Leica CM3050 S cryostat into 12 µm thick sections, and placed on SuperFrost Plus slides (Thermo Fisher Scientific, Waltham, MA, USA).

2.12 Immunostaining and imaging

Cryosection tissue slices were incubated with Leica Bond Wash solution (10X Bond wash, Leica Microsystems, Catalog No. AR9590, was used to prepare a 1 working solution in deionized water) for 3 minutes. Blocking for nonspecific staining was performed in 2 % normal goat serum with 1 % BSA solution applied at ambient temperature for 20 minutes, and then incubated with primary antibodies (150 µL/slide, Leica Microsystems M211518) for 30 minutes, followed by 3 washes with Leica Bond Wash solution. Thereafter, the slides were incubated with secondary antibodies with 1:2000 Hoechst (Sigma-Aldrich, St. Louis, MO, USA) for 20 minutes followed by 3 washes with Bond Wash Solution. For H&E staining, tissue slices were placed at room temperature for 10 mins. The sections then underwent standard haematoxylin and eosin (Thermo Fisher Scientific, Waltham, MA, USA) staining.

Photomicrographs were captured using Leica TCS SP8 MP multiphoton microscope and processed using Leica LAS X software. Objective lenses used included a 5X air objective and a 25X water objective. The z-stacks distance was adjusted using the auto Z steps function. Brightfield photographs (20X) were made with EVOS Life technology microscope. Statistical analyses were performed using GraphPad Prism.

3. Results

3.1 Bioprinted vascularized full-thickness skin equivalent (VFTS)

The basic features of the protocol for the 3D bioprinted vascularized skin tissue were: 1) preparation of a bioink made by mixing neonatal fibroblasts, pericytes, and human iPSC derived endothelial cells with a fibrin-based hydrogel; 2) bioprinting the bioink in the back of the customized transwell with a biodegradable PLGA scaffold to form a vascularized dermis (Fig. 1); 3) adding keratinocytes on the apical side of the scaffold on day 7 after bioprinting; and 4) performing ALI for 10-12 days for keratinocyte differentiation and epidermis maturation. The bioprinted human skin produced with this protocol has a layered architecture that consists of dermis and epidermis (Fig. 2a). In Fig. 2(a), the expression of KRT-10, filaggrin and loricrin demonstrated proper stratification and cornification, confirming full keratinocyte differentiation. High expression of desmoglein indicated cadherin junctions between keratinocytes. The integrin β1, laminin 5 and collagen IV were highlighted as indicators of basement membranes for keratinocytes and endothelial cells. The

basement membrane protein expressed around endothelial cell lumens demonstrated an integrated feature of stable microvascular network with ECM (Fig. 2f) [21,29]. 3D reconstructed confocal images provide additional evidence of the 3D vascularized dermal structure and epidermal layers showing the hierarchically ordered polygonal structure of different epidermal layers within a fully differentiated epidermis (Fig. 1c, Supplementary Video. 1). For functional validation, we used a high-throughput transepithelial resistance (TEER) measurement to assess the barrier function of the skin equivalents. The measurements from VFTS on ALI day 6, 8, 10 and 12 were $1667.4 \pm 386.5 \Omega \cdot \text{cm}^2$, $2716.6 \pm 545.9 \Omega \cdot \text{cm}^2$, $2835.2 \pm 1085.5 \Omega \cdot \text{cm}^2$, and $2132.6 \pm 596.8 \Omega \cdot \text{cm}^2$ (Fig. 2b), respectively, demonstrating high barrier integrity, and values above the threshold suggested by OECD guideline 430 ($500 \Omega \cdot \text{cm}^2$) [28].

The predefined geometry of 3D bioprinting structure contains initial acellular gel regions between the bioprinted strips of bioink (Fig. 1). We observed the tubulogenesis of endothelial cells within the printed structure around 24 hours after bioprinting, followed by the distinguished visualization of angiogenesis branching out from the printed structure to the cellular regions around one week after printing. The vascular bridging between printed structures became obvious after 17 days (Fig. 2d). The 3D reconstructed confocal images further confirmed the abundant blood vessel formations within the dermis (Fig. 2c, Supplementary Video. 1). The confocal images allow computational quantitative analysis by measuring intensity plots through the defined line probe.

3.2 Atopic dermatitis-like vascularized human skin equivalent and non-vascularized human skin equivalent

The elevated interleukin-4 (IL-4) secretion from T_H2 cells has been reported in lesional and non-lesional AD patients [30]. Treatment with IL-4 has been previously used to induce AD phenotypes in 3D skin equivalents of FTS or RhE [31]. Similarly, IL-4 treatment on our bioprinted FTS and VFTS tissues induces key AD phenotypes: spongiosis-like intercellular spaces, epithelial hyperplasia, and impaired differentiation (Fig. 3a). In the AD-like VFTS and FTS tissues, keratinocyte pericellular E-cadherin expression was significantly reduced compared to the control condition (Fig. 3a). The cleavage of E-cadherin in epidermis caused by the apoptosis of keratinocytes triggered the development of spongiosis [32]. Loricrin expression is also markedly reduced in AD-like equivalents, which is comparable to what is observed in acute non-lesional skin of AD subjects (Fig. 3a) [33]. TEER measurements of the tissue were taken during ALI days 6-10 (Fig. 3b). For tissues not treated with IL-4 (VEGF omitted), FTS (N=6) and VFTS tissues (N=8), TEER values of $2047.9 \pm 534 \Omega \cdot \text{cm}^2$ and $2815.7 \pm 822.7 \Omega \cdot \text{cm}^2$ on day 10 were obtained. The TEER values for FTS and VFTS

were substantially reduced in the IL-4 treated tissue to values of $176 \pm 49.8 \Omega \cdot \text{cm}^2$ and $567 \pm 534.4 \Omega \cdot \text{cm}^2$ after ALI day 10. No significant differences in TEER were observed between IL-4 untreated and treated RhE tissues, as reported previously (Supplementary Fig. 1) [34]. The differences in TEER seen upon IL-4 treatment for VFTS and FTS but not in RhE raises the question of whether simplified 3D skin tissues such as RhE are relevant models for inflammatory skin disease drug discovery. A panel of 21 cytokines and chemokines were measured from the cultured media at ALI day 8-10 (Supplementary Fig 2). Three cytokines, IL-1 α , serum thymus and activation regulated chemokine (TARC), and eotaxin were seen to increase significantly in the AD-VFTS and AD-FTS compared to control VFTS and FTS tissues (Fig. 4d). These cytokines have been reported to be upregulated in AD patients [35–37]. No increases in these cytokines were observed upon treatment of the RhE with IL-4 (Supplementary Fig. 1), once more highlighting that the incorporation of the dermis layer in the skin tissue equivalents produces a more physiologically relevant disease model.

3.3 JAK-STAT inhibition results in morphology and functional changes in AD-like skin equivalents

IL-4 activates the JAK-STAT pathway, which is known to upregulate epidermal chemokines, pro-inflammatory cytokines and pro-angiogenic factors (VEGF-A) in AD patients [38]. In pre-clinical testing and clinical trials, JAK inhibitors have recently shown efficacy for the treatment of AD [26,39–41]. We selected three JAK inhibitors currently in clinical trials for AD, ruxolitinib (NCT03011892), baricitinib (NCT03334396) and tofacitinib (NCT02001181) [42]. The addition of these JAK inhibitors (20 μM) improved epidermal morphology in both the AD-FTS and AD-VFTS models (Fig. 4a,b). The restored morphology and barrier function were accompanied by increased expression of differentiation proteins (fillagrin, KRT-10), cell-cell junctions (claudin-1) and dermal and epidermal junctions (DEJ) (integrin- β 1). JAK inhibitor tissue treatment also resulted in a significantly higher TEER value as compared to the AD controls (Fig. 4c). In FTS tissues, normal tissue has TEER value of $2048 \pm 534 \Omega \cdot \text{cm}^2$ (N=6), AD-like tissue has TEER value of $177 \pm 50 \Omega \cdot \text{cm}^2$ (N=6). For JAK inhibitor treated tissues, TEER for ruxolitinib is $3757 \pm 1449 \Omega \cdot \text{cm}^2$ (N=6), for baricitinib $2818 \pm 2186 \Omega \cdot \text{cm}^2$ (N=6), and for tofacitinib $2594 \pm 882 \Omega \cdot \text{cm}^2$ (N=6). Tissues treated with dexamethasone reached TEER value of $201.8 \pm 49.7 \Omega \cdot \text{cm}^2$ (N=6). In VFTS, controls show TEER of $1961 \pm 946 \Omega \cdot \text{cm}^2$ (N=17), AD-like of $472 \pm 468 \Omega \cdot \text{cm}^2$ (N=17). JAK inhibitors treated tissues show TEER values of $4354 \pm 2757 \Omega \cdot \text{cm}^2$ (N=9), $4300 \pm 2408 \Omega \cdot \text{cm}^2$ (N=9) $4790 \pm 2325 \Omega \cdot \text{cm}^2$, for (N=9) ruxolitinib, baricitinib and tofacitinib respectively. Dexamethasone treated tissue has TEER value of $298 \pm 175 \Omega \cdot \text{cm}^2$ (N=9). We further investigated the corrective effects of JAK inhibitors on the

cytokines elevated in the IL-4 induced AD skin tissue, IL-1 α , eotaxin, eotaxin-3, TARC, C-reactive protein (CRP), serum amyloid A (SAA), intercellular adhesion molecule-1 (ICAM-1) and vascular cell adhesion-1 (VCAM-1); were observed to be up-regulated compared to normal controls in our study and have been reported to be closely related to the severity of AD in patients (Fig. 4d, Supplementary Fig. 2) [43–47]. Our data also demonstrate that the above cytokines and chemokines are down regulated by all three types of the used JAK inhibitors. Notably, dexamethasone, an anti-inflammatory compound, did not rescue IL-4 induced phenotypes in either FTS or VFTS models, including morphological, later differentiation protein expression, tight junction barrier function and related chemokine and cytokine expression.

3.4 JAK inhibitors disrupt vasculature formation

We further studied the IL-4-induced phenotype of the dermal vasculature. A stripe patterned bioprinted geometry was created, using cell-containing hydrogel regions to facilitate visualization and quantification of angiogenesis, which is distinguished from the initial tube formation and vasculogenesis of endothelial cells within the printed region (Fig. 5a, g). IL-4 treatment selectively reduced angiogenesis while the vascular network was maintained within the printed structures (Fig. 5b, h). JAK inhibitors and dexamethasone treatment did not rescue the IL-4 reduced angiogenesis phenotype, and JAK inhibitors further reduced the vascular network in the bioprinted structure (Fig. 5c-f, i-l). Our results are consistent with previously reported results that JAK inhibitors have an anti-angiogenic effect [48]. The levels of VEGF-C, VEGF-D, ICAM-1 and VCAM-1 secretion from FTS and VFTS were downregulated by the JAK inhibitors but not by dexamethasone. Dexamethasone, ruxolitinib and tofacitinib significantly downregulated VEGF-A secretion in VFTS. These data indicate that the inhibition of JAK activity causes a potential side effect of disturbing dermal vascular stability and viability, while restoring morphology and barrier function of the skin tissue.

4. Discussion

Here, we developed a technique for the versatile and robust bioprinting of skin tissues with increased cellular complexity. The tissue, with proper morphology and barrier function, provides a platform for both topical and systemic drug discovery. Using the techniques described, we created an AD skin model, and used this model to test the effect of potential drug treatments for AD. The pharmacological responses obtained for these drugs in our system correlates with clinical therapeutic data. Our data further highlighted the importance of cellular complexity in 3D tissue disease modeling and drug screening.

A key feature of our biofabricated skin tissue is the incorporation of a biodegradable scaffold which provides a support for keratinocyte attachment, epidermis stratification in the tissue and could also enable a route for transplantation of this skin tissues into patients of skin diseases [25]. Another critical aspect of the protocol is the use of fibrin-based hydrogel which has been previously shown to prevent tissue contraction normally seen in traditional collagen-based skin constructs. In our biofabricated skin model, we successfully incorporated iPSC-derived endothelial cells into skin constructs. Treatment with VEGF and Angiopoietin-1 (ANG-1) promoted and stabilized tubulogenesis of ECs after one-week. VEGF is a well-known proangiogenic factor and ANG-1 is secreted by pericytes, which stabilizes vascular endothelial cells via Tie-2 receptor signaling pathways [49]. The first week submerged co-culture of vascularized dermis provides enough ECM development for keratinocyte differentiation and formation of a well stratified epidermis. This bioprinting protocol for the dermis enables the introduction of other cell types, such as T-cells, Langerhans cells, macrophages, nociceptors in the tissue. Finally, unlike the constructs built in customized microfluidic devices, the HTS-compatible multi-well dish format allows for quantitative measurements using existing HTS devices, for example, commercially available fully automated TEER machines, liquid handling devices, and automated liquid dispensing machines. This feature can increase the range of biological testings related to skin tissues.

According to the clinical studies, T_H2 cytokines, IL-4 and IL-13 are upregulated in AD patients. Compounds and biologics that target and inhibit IL-4 and IL-13 have been shown effective in clinical trials [50]. Manipulation of the culture process through supplementation of IL-4 during the ALI culture period, results in atopic dermatitis-like model that recapitulates the hyperplastic epidermis and impaired differentiation observed *in vivo*. For HTS quantification, TEER measurements show non-invasive advantages in the characterization of barrier functions[51]. In RhE models, we observed an increase in viable epidermis and formation of stratum corneum layer in IL-4-treated tissues without reduction in TEER values [52]. In FTS and VFTS models, we observed significant differences in TEER values from day 6 for VFTS models, and day 8 for FTS models between normal controls and IL-4 supplemented tissues. H&E stainings showed the viable epidermis thickness increased in both cases compared to their normal conditions. This accompanied with downregulation in E-cadherin and loricrin expression. We demonstrated that the degree of barrier differentiation protein expression impairment correlates with the decline in TEER values. In chronic inflammatory skin diseases, stimulated keratinocytes release IL-1, TNF- α and surface protein ICAM-1. The expression of ICAM-1 from RhE, FTS, and VFTS with supplements of IL-4 were observed significantly higher than

normal controls, which verified the association between the epidermal layer and ICAM-1. Additionally, IL-4 plays an important role in the upregulation of ICAM-1 in atopic skin [53,54]. IL-4 treatment induced VCAM-1 expression in FTS and VFTS AD-like tissue, but not in RhE tissues. The increased level of VCAM-1 and ICAM-1 is thought to be caused by the production of IL-4 and TNF- α . We did not observe upregulation of TNF α in any type of tissue in our system, this shows IL-4 itself can upregulate adhesion molecules, especially like VCAM-1 and ICAM-1.

In AD-VFTS, but not AD-FTS tissues, the expression of INF- γ increased significantly compared to the normal controls. Clinically, the production of cytokine is observed to predominantly shift from IL-4 in the initial stage to predominantly IFN- γ in the late and chronic phases [55]. In this case, including endothelial cells in skin equivalent is necessary in modeling chronic stage of AD[55,56]. Interestingly, cytokine levels of IL-1 α , eotaxin, and TARC significantly reduced with dexamethasone treated FTS tissue, but not in VFTS tissues compared to AD-FTS and AD-VFTS [57]. This highlights the inclusion of the vasculature changing the secretome of the skin tissue as it provides physiological relevant morphology. In the tissues treated with JAK inhibitors, microvessels got further collapsed or dissociated as the inhibitors were reported to have antiangiogenic effects and to reduce CD31 in tumor blood vessels [58]. The intervention of JAK-STAT signaling pathway further downregulated the expressions of most proangiogenic cytokines, and this antiangiogenic microenvironment destabilized vasculature. The observation of non-downregulated VEGF with the inhibitors in FTS will need further investigations to unravel the underlying mechanism of the compound reactions on multicellular communications in skin. (Fig. 5 a-m).

5. Conclusion

The bioprinting of human vascularized skin tissue in multi-well plates is a significant milestone for creating scalable high-throughput screening with complex physiologically relevant tissue models for drug discovery. Here it was shown that we can reproducibly biofabricated complex skin equivalents in multi-well plates. This reproducible complex 3D cellular culture enabled a high throughput pharmacological study in a truly physiologically relevant 3D tissue microenvironment for the first time. Further, the method developed here enables coupling of disease relevant multiplexed assay readouts and parallel therapeutic testing to establish both efficacy and possible side effects of treatments. This study also illustrates how data obtained using classic tissue models does not reflect more complex tissue interactions, and thus, results using these classic models should be interpreted with caution. The methodology described here is the first of its kind and is a step towards enabling the automated discovery of clinically relevant

compound leads with high potency and low side effects with high fidelity.

6. Acknowledgements

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Abbreviation list:

3D: Three dimensional
AD: Atopic Dermatitis
ALI: Air-liquid interface
ANG-1: Angiopoietin-1
BSA: Bovine serum albumin
CD31: Cluster of differentiation 31
CRP: C-reactive protein
DMEM: Dulbecco Modified Eagle Medium
EC: Endothelial cell
ECM: Extracellular matrix
FDM: Fully supplemented dermal medium
FTS: full-thickness skin
H&E: Hematoxylin and eosin
HTS: high throughput screening
ICAM-1: Intercellular adhesion molecule-1
IHC: Immunohistochemistry
IL-13: Interleukin 13
IL-1 α : interleukin 1 alpha
IL-4: Interleukin 4
INF- γ : Interferon gamma
iPSC: induced pluripotent stem cell
JAK: Janus kinases
KGF: Keratinocyte growth medium
KRT-10: keratin-10
MEM: Minimal essential medium
MSD: Meso Scale Discovery
NDFs: Neonatal human dermal fibroblasts
NHEKs: Neonatal human epidermal keratinocytes

OECD: Organization for Economic Co-operation and Development

PBS: Phosphate buffered saline

PLGA: poly(lactic-co-glycolic acid)

RhE: reconstructed human epidermis

SAA: Serum amyloid A

SD: standard deviation

STATs: Signal transducer and activator of transcription proteins

TARC: Serum thymus and activation regulated chemokine

TEER: Transepidermal electrical resistance

T_H2 cells: T helper type 2 cells

TNF- α : Tumor necrosis factor alpha

VCAM-1: Vascular cell adhesion-1

VEGF: Vascular endothelial growth factor

VFTS: vascularized full-thickness skin

Figure legends

Figure 1. Bioprinted vascularized skin and diseased condition. Bioprinting steps of vascularized full-thickness skin equivalent. Poly(lactic-co-glycolic acid) nanofibers scaffold was taken using Keyence 3D laser scanning confocal microscope (scale bar: 20 μ m). Vascularization in the dermis equivalent after one week of culture (CD31, red, Laminin, green, scale bar: 200 μ m).

Figure 2. Characterization of bioprinted vascularized skin tissue. (a) H&E staining of the tissue highlighted the architecture of the vascularized full-thickness skin (VFTS) tissue. Specific antibodies against human collagen IV, laminin 5, and integrin β proved the constituent of basement membrane. Expression of epidermal differentiation markers filaggrin, KRT10 and loricrin verified barrier formation of the VFTS tissue. (b) Functionality tests with TEER values of VFTS tissues over the air liquid interface (ALI) period of 6-12 days (N=10). (c) Reconstructed 3D VFTS tissue consists of fully developed vasculature with a fully stratified epidermis showing typical keratinocyte morphology and associated expression of differentiation markers (scale bar: 100 μ m). (d) A full view of microvasculature stained with CD31(red) showed angiogenesis in the dermis. (e) Microvessels demonstrated endothelial cell migration, sprouting and (f)the presence of laminin(yellow) proved maturation of the vessels.

Figure 3. Full-thickness skin equivalents (FTS) and vascularized full-thickness skin equivalents (VFTS) mimicking AD. (a) Histological characterization of normal and atopic dermatitis (AD)-like FTS and VFTS. Immunostaining for differentiation markers (loricrin) and cell-cell junction (E-cadherin). In contrast to control FTS and VFTS, the IL-4 exposed AD-like FTS and AD-like VFTS demonstrate thicker epidermis, intraepidermal edema with clear spaces separating keratinocytes and loss of terminal differentiation markers (scale bar: 50 μ m). (b) Tight junction barrier function integrity of normal and AD-like FTS over air liquid interface (ALI) culture of day 6-10 (N=6). TEER values of normal and AD-like VFTS over ALI culture period of day 6-10 (N=8). (c) Pro-inflammatory cytokine interleukin-1 α (IL-1 α), AD related chemokine, serum thymus and activation regulated chemokine(TARC) and eotaxin are compared between ALI day 1 and day 10 on normal and AD-like FTS and VFTS tissue(N=3). Tissues with normal conditions served as controls. 2way ANOVA test *P $\leq$ 0.05 compared to normal tissue. **P $\leq$ 0.01 compared to normal tissue. ***P $\leq$ 0.001 compared to normal tissue. ****P $\leq$ 0.0001 compared to normal tissue.

Figure 4. Effects JAK inhibitors on IL-4-exposed full-thickness skin equivalents (FTS) and vascularized full-thickness skin equivalents (VFTS)tissues. (a) FTS tissue with control, IL-4 treated, dexamethasone (20 μ M), baricitinib (20 μ M), ruxolitinib (20 μ M) and tofacitinib (20 μ M) treated conditions. JAK inhibitors were added simultaneously with IL-4 from air liquid interface (ALI) day 1. Presence of differentiation marker filaggrin in AD-like (IL-4 exposed) and dexamethasone-treated tissue is reduced compared with controls and JAK inhibitors treated tissues. Claudin-1 is dramatically reduced in the epidermis of AD tissues compared to controls and JAK inhibitors treated tissue. Suprabasal KRT-10 expression in the epidermis is typical in controls and in the presence of JAK inhibitors but scattered in AD-like and dexamethasone treated tissues, AD-like tissue has less expression of integrin- β 1, dexamethasone has multiple layers of integrin- β 1(scale bar: 50 μ m). (b) VFTS tissue with control, IL-4 treated, dexamethasone(20 μ M), baricitinib (20 μ M), ruxolitinib (20 μ M) and tofacitinib (20 μ M) conditions. Both H&E and phalloidin staining highlighted the architecture of the tissues. The integrin β 1 abundant expression due to the pericyte and endothelial cells angiogenesis in the vascularized dermis (scale bar: 50 μ m). (c) Comparison of tight junction barrier function of both FTS and VFTS with normal condition, AD condition, and JAK inhibitors treated tissues. (d) Effects of IL-4 and JAK inhibitors on cytokine and chemokine release of FTS and VFTS equivalents(N=3). Quantification of cytokines was performed from the medium collected from day 8-10. One-way ANOVA test *P $\leq$ 0.05 compared to AD-like tissue. **P $\leq$ 0.01 compared to AD-like tissue. ***P $\leq$ 0.001 compared to AD-like tissue. ****P $\leq$ 0.0001 compared to AD-like tissue.

Figure 5. Quantification of angiogenesis in vascularized full-thickness skin equivalents (VFTS) tissues. Confocal images of vascularized dermis were used to analyze angiogenesis of the tissues with different conditions. The tissues were harvested after 10 days of ALI. (a) normal VFTS angiogenesis highlighted with specific anti-CD31 in vascularized dermis. (b) Angiogenesis of AD-like tissue generated via with IL-4 exposure. (c) Dexamethasone treated tissue stained with CD31. Tissues stained with CD31 treated with JAK inhibitors of ruxolitinib (d), and tofacitinib (e), and baricitinib (f). (g), Intensity across the thick tissue sample inside the white lines shown in a. (h) AD-like VFTS intensity calculated use radius in (b). (i) CD-31 intensity across the radius of VFTS tissue treated with dexamethasone shown in (c). (j), CD-31 intensity across the radius of VFTS tissue treated with ruxolitinib shown in (d). (k), CD-31 intensity across the radius of VFTS tissue treated with tofacitinib shown in (e). (l), CD-31 intensity across the radius of VFTS tissue treated baricitinib shown in (f) (scale bar: 500 μ m). The calculation was done with MATLAB. (m), Cytokine related to vasculature measurement from both full-thickness skin equivalents (FTS) and VFTS tissue. The medium was collected on ALI day 10 (N=3). One-way ANOVA test *P $\leq$ 0.05 compared to AD-like

tissue. ** $P \leq 0.01$ compared to AD-like tissue. *** $P \leq 0.001$ compared to AD-like tissue. **** $P \leq 0.0001$ compared to AD-like tissue.

For the Supplementary data, please see other files.

Figure 1

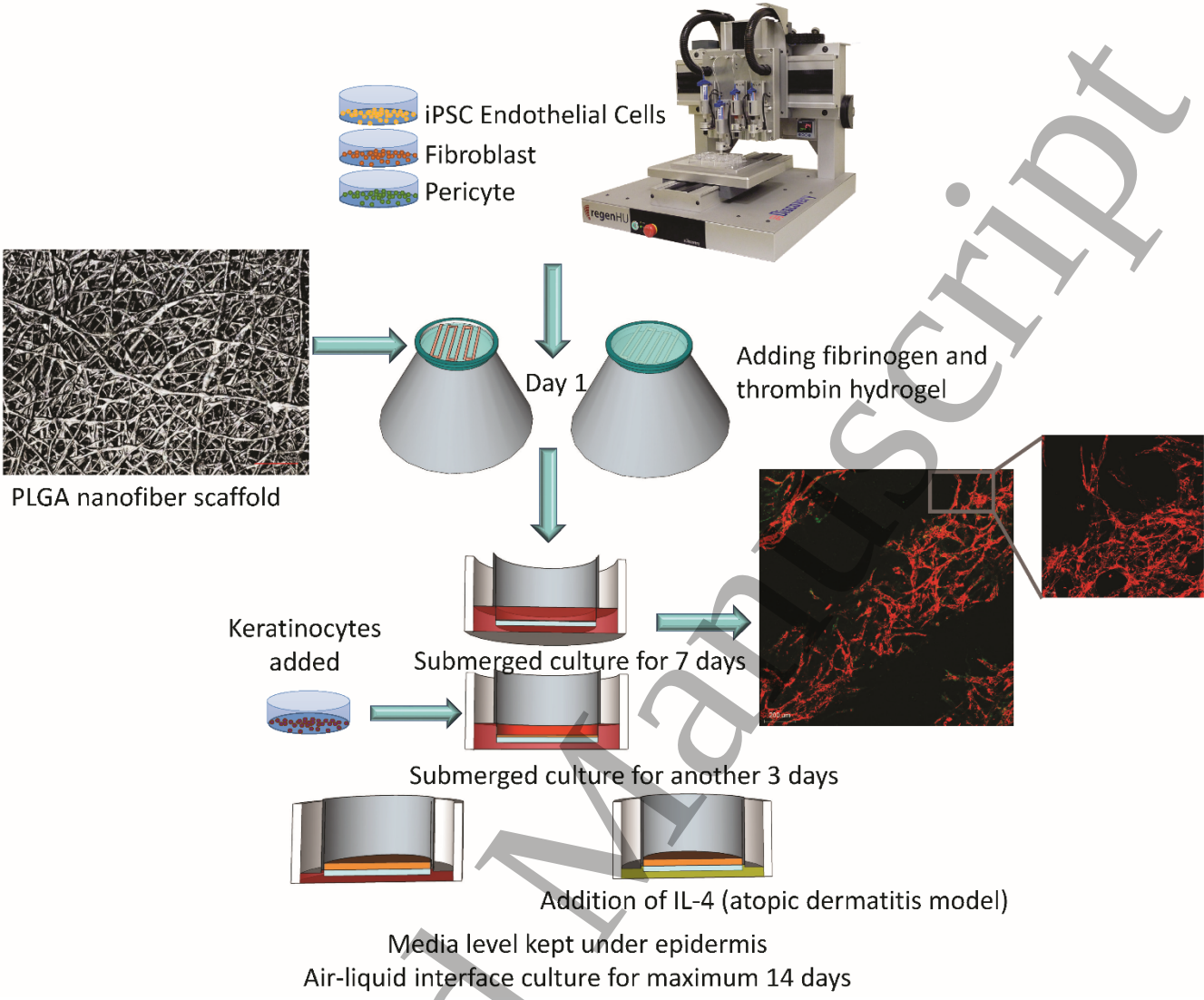


Figure 2

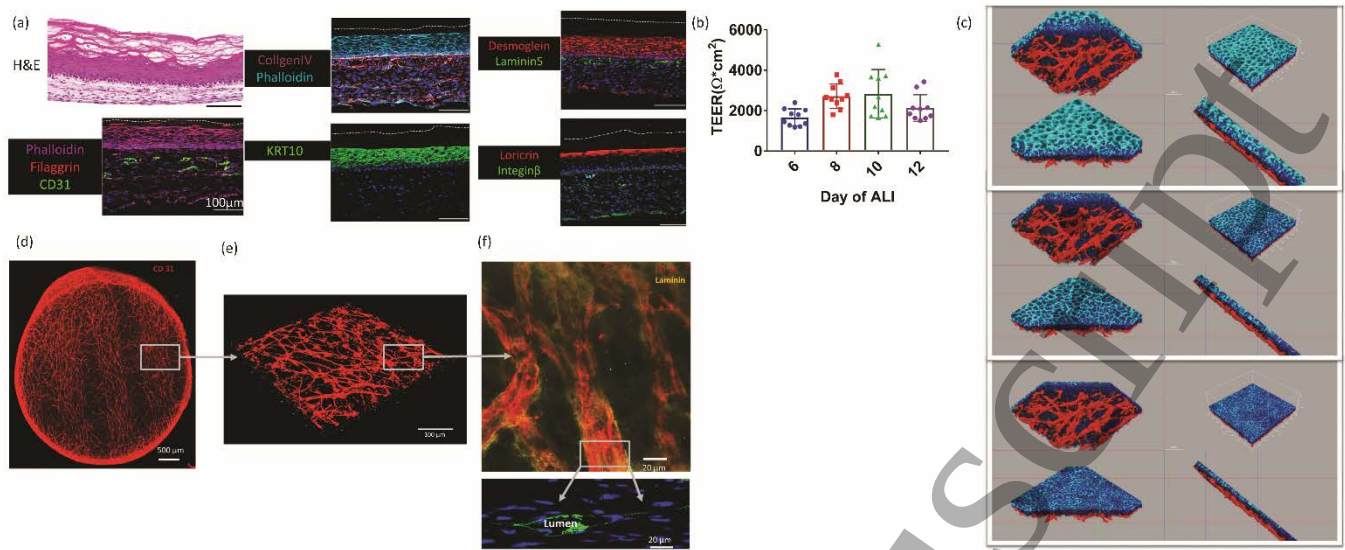


Figure 3

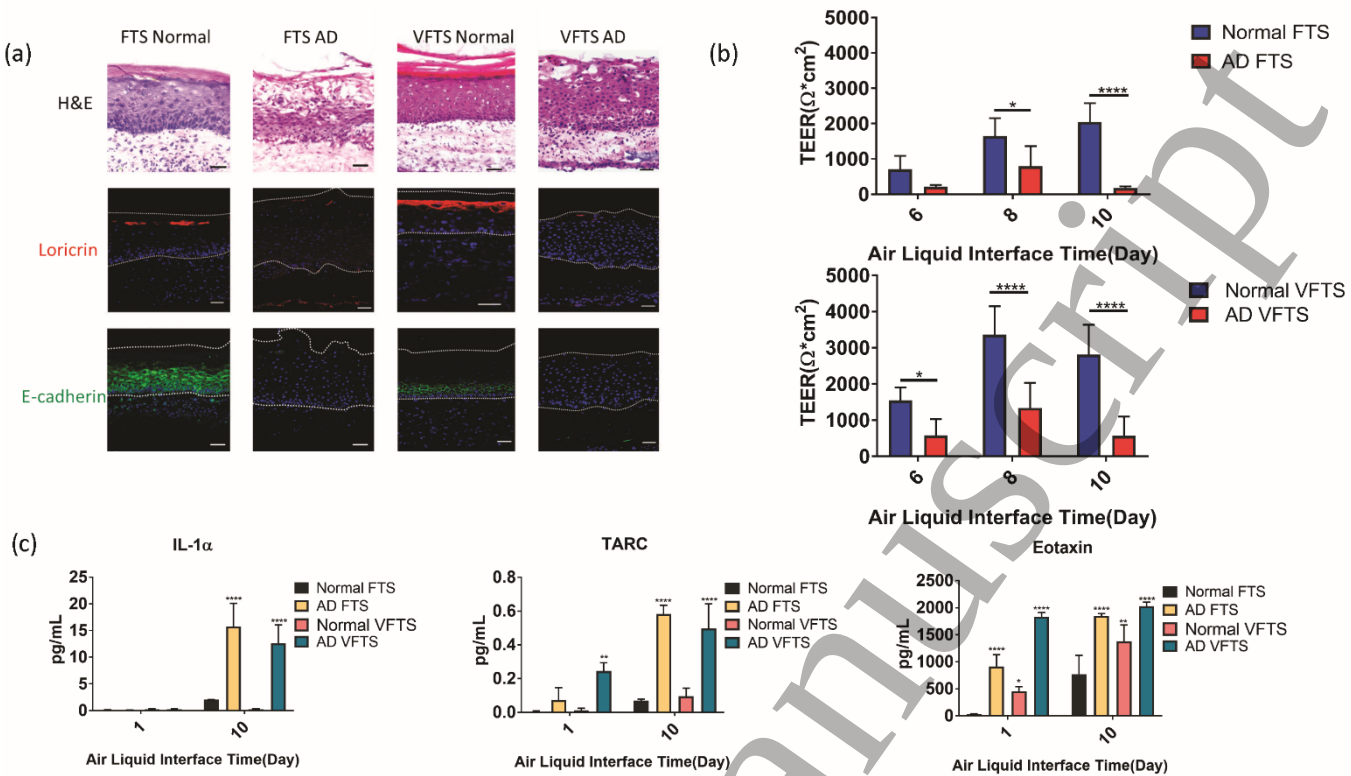


Figure 4

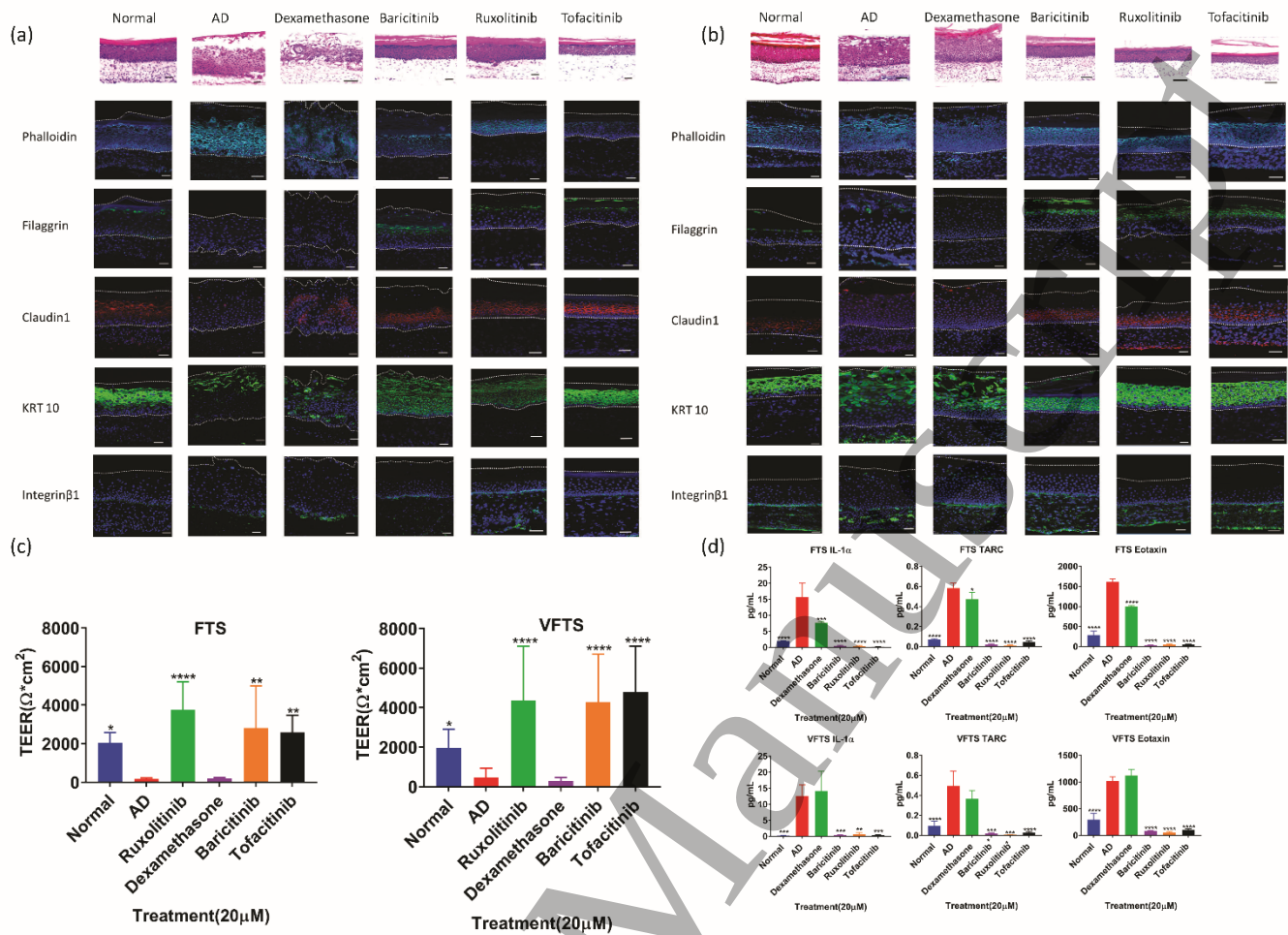
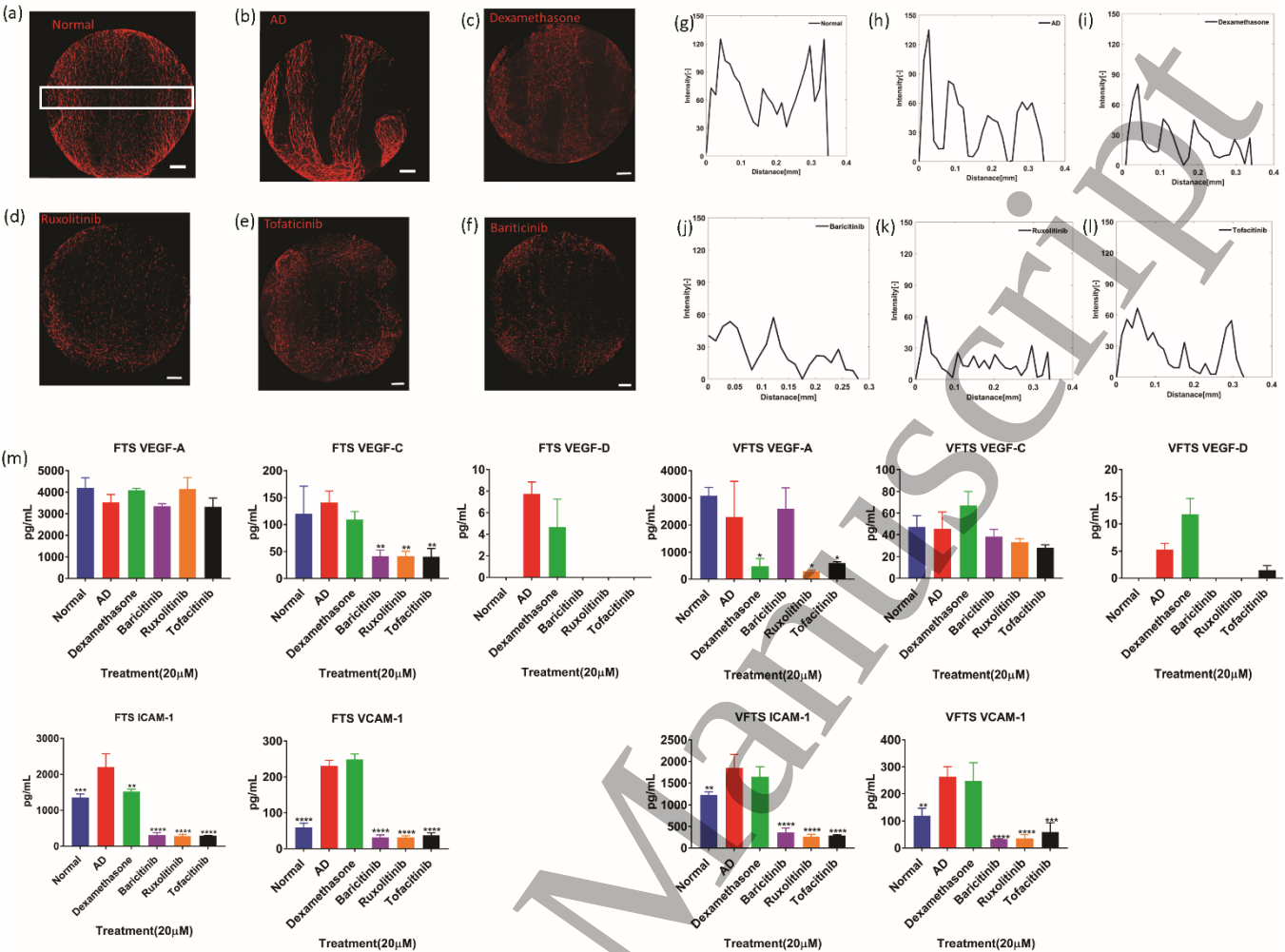
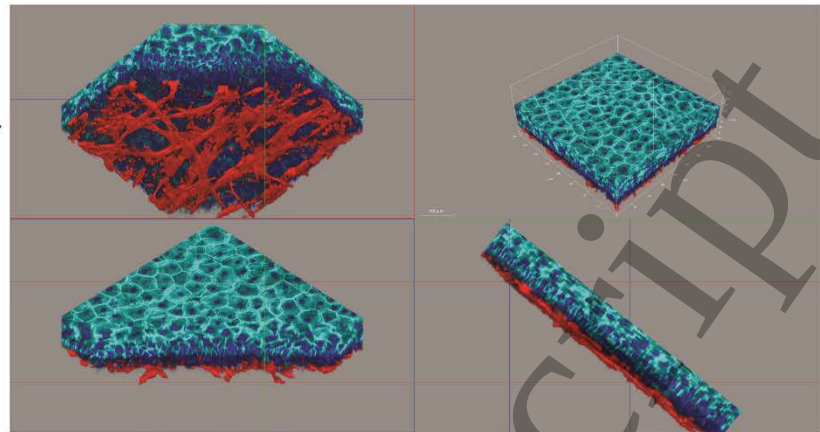
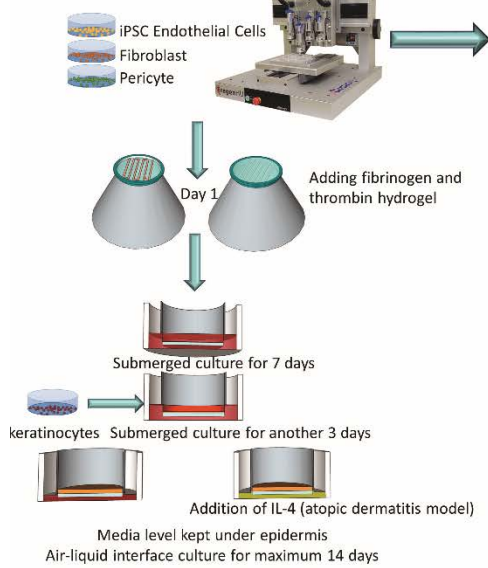


Figure 5



Graphical abstract

Bioprinted Vascularized Skin Equivalent



Generating AD-like skin equivalent and the tissue used for testing compounds

