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Research Article

3D CULTURE OF HaCaT KERATINOCYTE CELL LINE AS AN *in vitro* TOXICITY MODEL

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Abstract: *Ex vivo* dermal toxicology analyses are crucial for replacement of *in vivo* test methods and have been of interest in recent years for testing cosmetics, drugs, and chemicals. Development of an appropriate reconstructed epidermis model might overcome the limitations of monolayer culture systems. In the current study, we used the immortalized human keratinocyte cell line (HaCaT) to develop an *ex vivo* 3D cell culture system for keratinocyte-based toxicity analysis. Mouse embryonic fibroblast-conditioned medium and Matrigel matrix-based 3D HaCaT cell culture systems expressed skin-related genes and proteins in culture. The 3D HaCaT cultures demonstrated a skin-like phenotype and response against selected test compounds. Reliable results were obtained compared to monolayer HaCaT cells which were exposed to selected chemicals for 1 h and 24 h. Gene expression profiles of 3D HaCaT cell cultures and monolayer cultures were completely different after administration of the test compounds.
Overall, our results showed that a 3D HaCaT cell culture system generated in Matrigel matrix exerted a skin epidermis-like phenotype. Consequently, 3D HaCaT cell cultures may be an acceptable test method for conducting *in vitro* toxicology experiments.

Özet: *Ex vivo* dermal toksikoloji analizleri, in vivo test yöntemlerinin yerini alabilmesi için çok önemlidir ve son yıllarda kozmetik, ilaç ve kimyasalların test edilmesi için ilgi çekici hale gelmiştir. Kültürde yapılandırılmış uygun bir epidermis modelinin geliştirilmesi, tek katmanlı kültür sistemlerinin sınırlamalarını ortadan kaldırabilir. Bu amaçla, mevcut çalışmada, keratinosit bazlı toksisite analizi amacı ile bir *ex vivo* 3D hücre kültürü sistemi geliştirmek için ölümsüzleştirilmiş insan keratinosit hücre hattı (HaCaT) kullandık. Fare embriyonik fibroblast kondisyonel besiyeri ve Matrigel matriksi tabanlı 3D HaCaT hücre kültürü sistemleri, kültürde deri ilgili genleri ve proteinleri ifade etti. 3D HaCaT kültürleri, deri benzeri bir fenotip gösterdi ve seçilen test bileşiklerine karşı tepki gösterdi. 1 saat ve 24 saat boyunca seçilen kimyasallara maruz bırakılan tek katmanlı HaCaT hücrelerine kıyasla güvenilir sonuçlar elde edildi. 3D HaCaT hücre kültürlerinin ve tek tabakalı kültürlerin gen ekspresyon profilleri, test bileşiklerinin uygulanmasından sonra tamamen farklıydı.
Özet olarak, sonuçlarımız Matrigel matrisinde oluşturulan bir 3D HaCaT hücre kültürü sisteminin cilt epidermisi benzeri bir fenotip uyguladığını gösterdi. Sonuç olarak, 3D HaCaT hücre kültürleri, *in vitro* toksikoloji deneylerinin yürütülmesi için kabul edilebilir bir test yöntemi olabilir.

Introduction

Skin irritation testing is a common part of the biological safety evaluation of many materials and products. Skin irritation analyses have been performed using various laboratory animal models, including rabbits (Miles *et al.* 2014).

The skin irritation potential of chemicals, cosmetics, drugs, and medical devices can be evaluated *in vitro* with reconstructed human epidermis (RhE) model systems. Replacement of animal models with *in vitro* cellular systems has been increasing in recent years due to various testing bans (Pellevoisin *et al.* 2018), ethical considerations, handling and cost problems.

Restriction of animal use in skin toxicity tests reduces animal discomfort and undesired pain due to skin

irritation analysis. Establishment of human tissue-based *in vitro* model systems is not only important for animal well-being, but also eliminates species-specific differences between human and animal models.

Cosmetic regulations restricting the use of animals for toxicology testing (Almeida *et al.* 2017) drove the development of alternative methods, which was an important step in the creation of reconstructed human skin models. Development of appropriate models and validation of these models might be a solution in the near future for skin toxicology analysis.

Keratinocytes are the primary cells of the human epidermis (Colombo *et al.* 2017). In addition to providing the epidermal barrier function, keratinocytes play a role in the inflammatory response of skin (Hänel *et al.* 2013, Colombo *et al.* 2017) and wound repair processes in the re-epithelization step (Pastar *et al.* 2014). Establishment of the immortalized human keratinocyte cell line (HaCaT) eliminated the problems observed with primary keratinocytes such as their need for growth supplements, culture differentiation, donor-related variability and limited lifespan. HaCaT cells have been used for many skin model studies without altering the keratinocyte function, cellular responses or differentiation capacity (Schürer *et al.* 1993, Garach-Jehoshua *et al.* 1998, Micallef *et al.* 2009). Monolayer HaCaT cell cultures are used for many application such as cellular toxicity and *in vitro* wound healing analysis. However, monolayer culture systems cannot totally replicate the actual epidermal structure. Though, generation of an air-liquid interface in keratinocyte cell cultures might yield a more realistic model of an epidermis (do Nascimento Pedrosa *et al.* 2017). Using immortalized HaCaT cells to form 3D skin models was successful as a constructed skin model for safety evaluation (Mini *et al.* 2021). Although monolayer 2D HaCaT cell cultures can be used for cell proliferation, viability and toxicity analysis, 3D culture systems are more appropriate for establishing an epidermis-like structure for *in vitro* testing.

In the present study, we developed an extracellular matrix-based 3D model system using HaCaT cells. We evaluated the model's protein secretion profile and skin related gene expression to determine if it would be an applicable *in vitro* assay for epidermal toxicity studies.

Materials and Methods

HaCaT Cell Culture

Human keratinocyte cell line HaCaT (CLS 300493, DKFZ, Heidelberg, Germany) was cultured in Dulbecco's Modified Essential Medium (DMEM) containing 10% fetal bovine serum (FBS) (Invitrogen, Waltham, MA, USA), and 1% of penicillin, streptomycin, and amphotericin B (PSA) (Invitrogen, Waltham, MA, USA). Cells were incubated in a humidified chamber at 37°C and 5% CO_2 until 80% confluency was reached.

Mouse Embryonic Fibroblast (MEF) Isolation and Culture

Mouse Embryonic Fibroblast (MEF) cells were isolated as described previously (Şişli *et al.* 2021). Mice were housed at a constant temperature (23 ± 1°C) with humidity (60 ± 10%), and subjected to an artificial 12 h light/dark cycle and fed with food and water ad libitum. Animal handling and surgical procedures were approved with an ethical permission obtained from the Yeditepe University Ethics Committee of Experimental Animal Use and the Research Scientific Committee of Yeditepe University-Türkiye (21.12.2018-No:714 ethical approval). Briefly, one pregnant BALB/c mouse at day 11-14 post-coitum was euthanized and disinfected with 70% ethanol prior to necropsy. The head and inner organs were removed, minced into small pieces, and incubated in Trypsin (Gibco, Waltham, MA, USA). MEFs were separated from tissue pieces by incubation at 37°C and plated onto T150 flasks (TPP, Trasadingen, Switzerland). The MEFs were cultured in DMEM containing 10% FBS, and 1% PSA. Cells were cultured in a humidified incubator at 37°C and 5% CO_2 until 80% confluency.

Collection of Conditioned Medium from MEFs

MEFs (5×10^5) were seeded in 100 mm culture dish (TPP, Trasadingen, Switzerland) in 10 ml complete DMEM medium and incubated 1 day in incubator. Culture medium was replaced with a fresh medium 24 h before conditioned medium collection. Conditioned medium was collected from cells at 80% confluency. Collected medium was centrifuged and filtered through a 0.22 µm syringe filter (GVS SpA, Bologna, Italy) to remove any cellular debris. Conditioned medium was aliquoted and stored at -20°C until the analysis of the angiogenesis protein array (Fig. 1a). Conditioned medium of MEFs was used to promote cell proliferation and growth of HaCaT cells by providing growth factors and cytokines released from MEFs into the conditioned medium.

3D Culture of HaCaT Cells

HaCaT cells were cultured in Matrigel basement matrix (Corning Life Sciences, Lowell, MA, USA) to generate a 3D epidermis model for toxicity analysis. Culture medium was prepared according to the previously published protocol with minor modifications (do Nascimento Pedrosa *et al.* 2017). Culture medium content is shown in Table 1 for monolayer and Matrigel based 3D HaCaT cell cultures.

A 0.4 µm pore sized 24-well plate insert (Corning Life Sciences, Lowell, MA, USA) was used to culture HaCaT cells in Matrigel basement matrix. HaCaT cells at a cell density of 1×10^5 cells/insert were mixed with Matrigel-medium mixture (1:1, 100 µl medium+100 µl Matrigel) and placed on the top of the inserts. Inserts were incubated for 30 min with humidity at 37°C to allow for Matrigel polymerization. Approximately 500 µl of medium was added into the bottom part of the 24-well plates (TPP, Trasadingen, Switzerland) to cover the insert and generate

an air-liquid interface. The plates were incubated for 9–11 days prior to use and the medium was changed every two days (Fig. 2a).

In vitro Toxicity Analysis

In vitro cellular toxicology analyses were performed by using standard monolayer culture conditions and 3D HaCaT cell cultures to observe applicability of the technique. Culture conditions and concentrations of selected chemicals are shown in Table 2.

Selected chemicals were freshly prepared in DMEM medium and administered to the 3D HaCaT cell cultures for 1 h and 24 h (Fig. 3a). Time points were selected to analyze short-term and long-term effects of the selected chemicals on the generated model. Media containing the tested compounds were removed; inserts were washed with PBS (Gibco, Waltham, MA, USA) and subjected to cell viability analysis.

Two different incubation times were selected for 2D monolayer cultures. Cells were seeded as 4×10^4 number of cells/well and incubated 24 h with humidity. On the following day, the tested compounds were administered for 1 h and 24 h for toxicity analysis (Fig. 3B). At the end of incubation time, cell viability analyses were performed. Cell viability was measured by MTS assay (Demirci *et al.* 2016). The MTS reagent (10%) prepared in complete growth medium was applied to the cells and incubated for 2 h at 37°C. The absorbance was measured at 490 nm using an ELISA plate reader (BioTek, Winooski, VT). Absorbance values were used as an indicator of cell viability and toxicity. Cell viability was compared to negative control and positive control chemicals. Loss of cell viability was used as an indicator of keratinocyte toxicity.

Table 1. 2D and 3D culture medium content.

	Medium Content
2D monolayer culture conditions	- DMEM -1% antibiotics -10% FBS
3D monolayer culture conditions	-DMEM/F12 -5 µg/mL insulin -0.4 µg/mL hydrocortisone -5 µg/mL transferrin -2 ng/mL TGF-β -1 ng/mL EGF -5% of conditioned medium obtained from MEFs cultured for 24 h at high confluence (~80%). -1% antibiotics -10% FBS

DMEM: Dulbecco's Modified Essential Medium, FBS: Fetal Bovine Serum, TGF-β: Transforming Growth Factor-Beta, EGF: Epidermal Growth Factor, MEF: Mouse Embryonic Fibroblasts

Table 2. Culture conditions and concentrations of selected chemicals.

	Representations of the Chemicals	Chemicals for Toxicity Analysis	CAS number
1	NC	Cell culture media	-
2	PBS	DPBS	-
3	IPA	2-propanol	67-63-0
4	SDS (PC)	5% SDS	151-21-3
5	KOH	5% KOH	1310-58-3
6	NaOH	0.5M NaOH	1310-73-2
7	Ba High	1000 µg/mL BA	10043-35-3
8	Ba Low	100 µg/L BA	
9	NaB High	1000 µg/mL NaB	12007-92-0
10	NaB Low	100 µg/L NaB	
11	Oleic Acid High	1000 µg/mL Oleic Acid	112-80-1
12	Oleic Acid Low	100 µg/L Oleic Acid	

DPBS: Dulbecco's phosphate-buffered saline, 2-propanol: isopropyl alcohol, SDS: Sodium Dodecyl Sulphate, KOH: Potassium hydroxide, NaOH: Sodium hydroxide, BA: Boric Acid, NaB: Sodium pentaborate decahydrate

Gene expression analysis

Primers (Table 3) were designed using Primer-BLAST software from the National Center for Biotechnology (Bethesda, MD, USA) and synthesized by Sentegen (Ankara, Turkey). β-Actin was used as a housekeeping gene. Total RNAs were isolated using RNeasy plus mini kit (Qiagen, Hilden, Germany) following the manufacturer's instructions.

Table 3. Primers used in qPCR analysis

Gene	Sequence
COL1A	F 5' CCACGCATGAGCGGACGCTAA 3' R 5' ATTGGTGGGATGTC TTCGTCTTGG 3'
EGF	F 5' CACACTGGAAAGGACATGGT 3' R 5' TAACATCAGGCTCCCAAGT 3'
FGF2	F 5' GGTGAsAACCCCGTCTCTACA 3' R 5' TCTGTTGCCTAGGCTGGACT 3'
FGF7	F 5' CTCAAGCACTCGGGGATAAA 3' R 5' CTGTTTTGGCAGGACAGTGA 3'
Fibronectin	F 5' AGCCTGGGAGCTCTATTCCA 3' R 5' CTTGGTCGTACACCCAGCTT 3'
HB-EGF	F 5' CGGCAGCAACCAGCAACCC 3' R 5' GAGCGCGATCAGCTCCCGTG 3'
IL-8	F 5' GCAGAGGGTTGTGGAGAAGT 3' R 5' ACCCTACAACAGACCCACAC 3'
IL-10	F 5' GACTTTAAGGGTTACCTGGGTTG 3' R 5' TCACATGCGCCTTGATGTCTG 3'
MMP-2	F 5' ATCCAGTTTGGTGTCGCGGAGC 3' R 5' GAAGGGGAAGACGCACAGCT 3'
MMP-9	F 5' GGCCCTGTCACTCCTGAGAT 3' R 5' GCATCCAGGTTATCGGGGA 3'
TGF-β1	F 5' CAAGTGGACATCAACGGGT 3' R 5' CCGTGGAGCTGAAGCAATA 3'
VEGF	F 5' CACCATGCAGATTATGCGGA 3' R 5' GTTGTGCTGTAGGAAGCTCA 3'
VEGFR1	F 5' GCTCAGCTGTCTGCTTCTCA 3' R 5' ATTTATGGGCTGCTTCCCCC 3'
VEGFR2	F 5' CGTGTCTTTGTGGTGCACTG 3' R 5' TCTAGGACTGTGAGCTGCCT 3'
β- Actin	F 5' GACAGGATGCAGAAGGAGATTACT 3' R 5' TGATCCACATCTGCTGGAAGGT 3'

iScprit cDNA synthesis kit (Bio-Rad Laboratories, Inc., Hercules, CA, USA) was utilized for cDNA synthesis. SYBR Green (Applied Biosystems, Waltham, MA, USA) qPCR method was used as described previously. CFX96 RT-PCR system (Bio-Rad Laboratories, Inc., Hercules, CA, USA) was used in all RT-PCR experiments.

Protein array analysis

Angiogenesis Antibody Array (#AAH-ANG-1-8, Ray Biotech, Peachtree Corners, GA, USA), and Growth Factor Antibody Array C1 (#AAH-GF-1-8, RayBiotech, Peachtree Corners, GA USA) were used to determine protein expression profile of MEFs and 3D HaCaT cell cultures, respectively. Briefly, proteins were isolated by the array kit's lysis buffer and protein concentrations were measured by bicinchoninic acid (BCA) (ThermoScientific, Waltham, MA, USA) assay. Membranes were incubated in a blocking buffer for 30 min and then treated with protein samples overnight at +4°C on a rocking platform. On the following day, membranes were washed 3 times with wash buffer and incubated with biotinylated antibody for 2 h at room temperature. Membranes were washed, treated with horseradish peroxidase (HRP) for 2 h and protein expression was detected and visualized by a luminometer system (ChemiDoc XRS, Bio-Rad Laboratories, Inc., Hercules, CA, USA). Protein spot intensity measurements were conducted by Image Lab software (Bio-Rad Laboratories, Inc., Hercules, CA, USA).

CoL1a1 ELISA

Protein from 3D HaCaT cell cultures and a monolayer cell culture were isolated by RIPA Lysis Buffer (Santa Cruz Biotechnology, Inc., Dallas, TX, USA) and protein concentrations were measured by BCA (ThermoScientific, Waltham, MA, USA) assay. A CoL1a1 ELISA (#ab210966, Abcam plc, Cambridge, UK) was performed according to the manufacturer's instructions. Briefly, protein extracts were diluted 5-fold and applied to the wells of ELISA strips along with standards. An antibody cocktail was subsequently added to the same wells. The plate was incubated for 1 h at room temperature on a plate shaker. Then the wells were washed 3 times in 3,3′,5,5′-tetramethylbenzidine. Developing solution was then added to the protein sandwich. To halt the reaction, stop solution was added to the wells and absorbance was measured by an ELISA plate reader (BioTek, Santa Clara, CA, USA) at 450 nm. A standard curve method was used to calculate amounts of CoL1a1 protein.

Statistical analysis

Experiments were conducted in triplicate (3 replicates in each experiment) and statistical analyses were performed using one-way analysis of variance (ANOVA) post hoc Tukey test. P values less than 0.05 were considered statistically significant. All statistical analysis and correlation analysis were performed using the GraphPad Prism version 9.0 for Windows (GraphPad Software, Inc., San Diego, CA, USA).

Results

MEF cell conditioned medium contains growth factors and cytokines

MEF cell conditioned medium was used in the current study to provide appropriate growth factors and cytokines for skin-like structure formation. MEF cells with spindle-like cellular morphology were successfully isolated, and the conditioned medium collected from MEFs was used for protein array analysis (Fig. 1a). A panel of 20 proteins including GRO (α, β, γ), PLGF, angiogenin, IL-8, TIMP2, IFN-γ, RANTES, EGF, leptin, TPO, IGF-1, TNF-β, ENA78, MCP-1, VEGF-A, IL-6, TIMP1, bFGF, PDGF-BB and VEGF-D were detected by protein membrane array analysis (Fig. 1b). Angiogenin expression was not detected in the MEF conditioned medium, however, all the other growth factors and cytokines were detected. RANTES, TIMP1, VEGF-A and VEGF-D were specifically upregulated compared to the negative control (Fig. 1b).

3D HaCaT cell cultures demonstrated skin-like gene and protein expression profile

The HaCaT cells were cultured in insert well systems with Matrigel basement membranes (Fig. 2a). HaCaT cells demonstrated standard keratinocyte morphology in 2D monolayer culture conditions. HaCaT cells were maintained in Matrigel-based 3D culture in insert wells and remained intact structures for 10 days (Fig. 2b). Cells expressed a number of skin related genes including bFGF, Col1a, EGF, FGF7, Fibronectin, HB-EGF, IL-10, IL-8, MMP2, MMP9, TGFβ1, VEGF, VEGFRI, VEGFRII (Fig. 2c). Fibronectin, MMP2, TGFβ1 and VEGF gene expression levels were significantly upregulated in 3D HaCaT cell cultures compared to 2D monolayer culture system (Fig. 2c).

The 3D HaCaT cell cultures generated in Matrigel matrix expressed many growth factors which are important for skin biogenesis and formation (Fig. 2d). IGFBP-2, IGFBP-6, M-CSFR, PDGF-AA and EGFR protein expression levels were upregulated among others in 3D HaCaT cell cultures compared to negative controls (Figs 2e-f**)**. CoL1a1 protein expression was detected in monolayer and 3D HaCaT cell cultures by ELISA measurement. 3D HaCaT cell cultures demonstrated high expression of CoL1a1 protein indicating the potential for its use as an epidermis model (Fig. 2g).

3D HaCaT cell cultures are more suitable for in vitro toxicology analysis

3D HaCaT cell cultures and monolayer culture systems were used for *in vitro* toxicology testing to evaluate the applicability of the systems. The 3D HaCaT cell cultures were dosed with test compounds and incubated for 1 h and 24 h to simulate skin toxicity conditions (Fig. 3a). Monolayer culture systems were also dosed and incubated for 1 h and 24 h exposure times and the results were compared with the

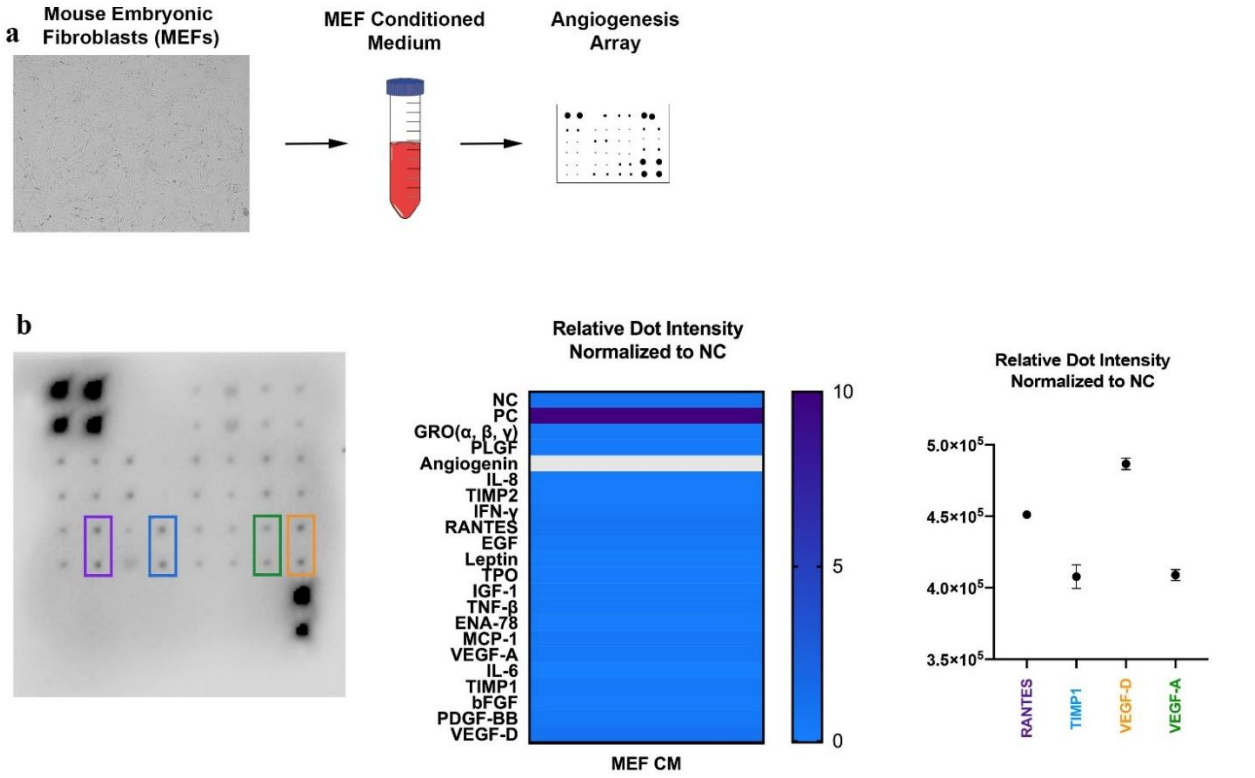

Fig. 1. Isolation of mouse embryonic fibroblast cells (MEFs) and collection of conditioned medium. **a.** Morphological appearance of MEFs and experimental flow, **b.** protein membrane array analysis, heat map representation and significantly upregulated proteins. NC: Negative Control of Angiogenesis Array, PC: Positive Control of Angiogenesis Array, GRO (α,β,γ): growth regulated gene-alpha-beta-gamma, PLGF: Placental growth factor, IL-8: Interleuikin-8, TIMP2: Tissue inhibitor of metalloproteinases 2, IFN- γ: Interferon-gamma, EGF: Epidermal growth factor, TPO: thyroperoxidase, IGF-1: Insulin growth factor-1, TNF- β: Tumor necrosis factor-beta, ENA-78: Epithelial cell-derived neutrophil-activating peptide-78, MCP-1: Monocyte chemoattractant protein-1, VEGF-A: Vascular endothelial growth factor A, IL-6: Interleukin-6, TIMP1: Tissue inhibitor of metalloproteinases 1, bFGF: basic fibroblast growth factor, PDGF-BB: platelet-derived growth factor-BB, VEGF-D: Vascular endothelial growth factor D.

3D HaCaT cell culture results (Fig. 3b). Cellular morphological analysis clearly showed the cellular response of monolayer HaCaT cells against toxic chemicals such as IPA, KOH, NaOH and SDS (Fig. 3c). Cell viability analysis were performed for 1 h and 24 h monolayer cell cultures and 1 h 3D HaCaT cell cultures. Results demonstrated that 3D HaCaT cell cultures produced a more skin-like response compared to monolayer HaCaT cells. Although PBS was not toxic on 3D HaCaT cell cultures, cell viability was significantly reduced in monolayer cultures during 1 h and 24 h PBS administration. Similar results were obtained for boron compound applications. The 3D HaCaT cell cultures did not show any toxicity from exposure to boron compounds, but the same compounds produced statistically significant toxicity in monolayer systems (Fig. 3d). As expected, IPA, KOH, NaOH and SDS were toxic in both experimental systems. Oleic acid showed dose-dependent toxicity (e.g., low concentrations of oleic acid did not produce any toxicity in 3D and monolayer HaCaT cell cultures, but high concentrations of oleic acid caused toxicity (Figs 3c-d).

Variance in gene expression patterns of 3D and monolayer HaCaT cell cultures

The bFGF, Col1a, EGF, IL-8, MMP9, TGFβ1, VEGF, and VEGFRI genes were used to identify the response of cell culture systems and administration routes. The 3D HaCaT cell cultures and monolayer culture systems responded differently against boron compounds, PBS and IPA. In addition, 1 h and 24 h treatments of monolayer culture systems produced different results indicating the importance of 3D HaCaT cell cultures (Fig. 4a). Heat map representations of qPCR analysis showed that 3D HaCaT cell cultures and monolayer culture systems displayed different gene expression patterns for each gene (Fig. 4a). Correlation matrix analysis demonstrated that 3D HaCaT cell cultures and monolayer culture systems had distinct gene expression profiles with specific correlation coefficient values after 1 h (Fig. 4b**)** and 24 h (Fig. 4c) selected compound exposure. Interestingly, PBS and IPA resulted in a similar gene expression profile according to correlation matrix in monolayer HaCaT cells after 1h exposure. However, PBS and IPA did not have a similarity with a coefficient value below zero in 3D HaCaT cell cultures, which is more expected and reliable for a toxicity analysis (Fig. 4b). In contrast, in 3D HaCaT cell culture for 24h exposure time, PBS and IPA had very similar coefficient values approximately 1.00 (Fig. 4c).

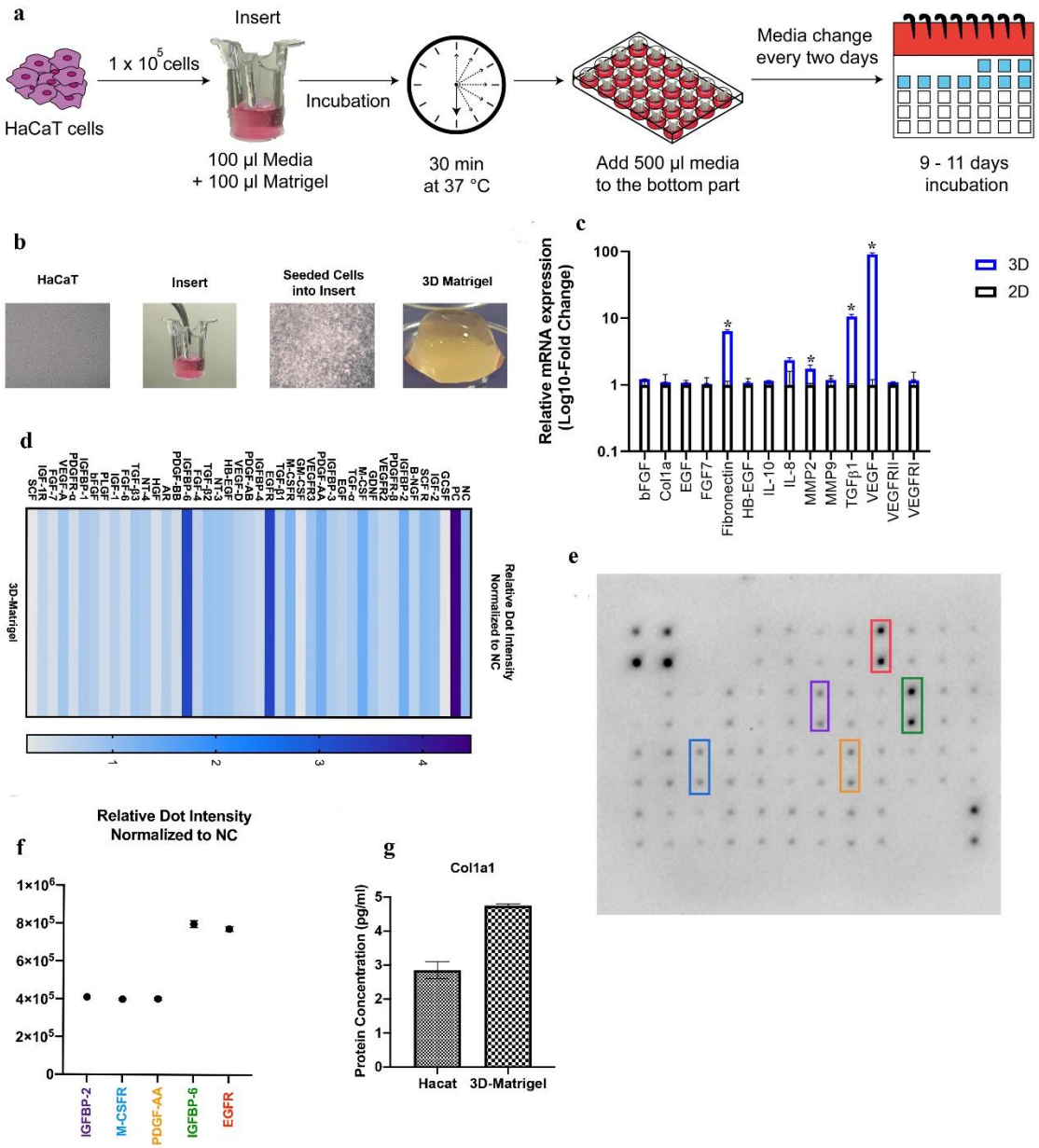

Fig. 2. Construction of 3D HaCaT cell cultures. **a.** Experimental flow of generation of 3D HaCaT cell cultures, **b.** morphological appearance of 2D and 3D HaCaT cell cultures, **c.** gene expression profile of 2D and 3D HaCaT cell cultures, **d.** heat map representation of 3D HaCaT cell culture protein array analysis, **e.** protein membrane analysis and **f.** significantly upregulated proteins, **g.** col1a1 protein expression of 2D and 3D HaCaT cell cultures. *$p < 0.05$. NC: Negative Control of Angiogenesis Array, PC: Positive Control of Angiogenesis Array, GCSF: Granulocyte colony-stimulating factor, IGF-2: Insulin growth factor-2, SCFR: Stem cell growth factor receptor, B-NGF: Beta-Nerve growth factor, IGFBP-2: Insulin-like growth factor-binding protein-2, PDGFRβ: Platelet-derived growth factor receptor beta, VEGFR2: Vascular endothelial growth factor receptor-2, GDNF: Glial cell-derived neurotrophic factor, M-CSF: Macrophage colony-stimulating factor, TGFα: Transforming growth factor-alpha, EGF: Epidermal growth factor, IGFBP-3: Insulin-like growth factor-binding protein-3, PDGF-AA: platelet-derived growth factor-AA, VEGFR3: Vascular endothelial growth factor receptor-3, GM-CSF: Granulocyte-macrophage colony-stimulating factor, M-CSFR: Macrophage colony-stimulating factor receptor, TGFβ1: Transforming growth factor-β1, EGFR: Epidermal growth factor receptor, IGFBP-4: Insulin-like growth factor-binding protein-4, PDGF-AB: platelet-derived growth factor-AB, VEGF-D: Vascular endothelial growth factor D, HB-EGF: Heparin-binding EGF-like growth factor, NT-3: Neurotrophin-3, TGFβ2: Transforming growth factor-β2, FGF4: Fibroblast growth factor 4, IGFBP-6: Insulin-like growth factor-binding protein-6, PDGF-BB: platelet-derived growth factor-BB, AR: Androgen receptor, HGF: Hepatocyte growth factor, NT-4: Neurotrophin-4, TGFβ3: Transforming growth factor-β3, FGF6: Fibroblast growth factor 6, IGF-1: Insulin growth factor-1, PLGF: Placental growth factor, bFGF: basic fibroblast growth factor, IGFBP-1: Insulin-like growth factor-binding protein-1, PDGFRα: platelet-derived growth factor receptor alpha, VEGF-A: Vascular endothelial growth factor-A, FGF-7: Fibroblast growth factor-7, IGF-1R: Insulin growth factor-1 receptor, SCF: Stem cell factor.

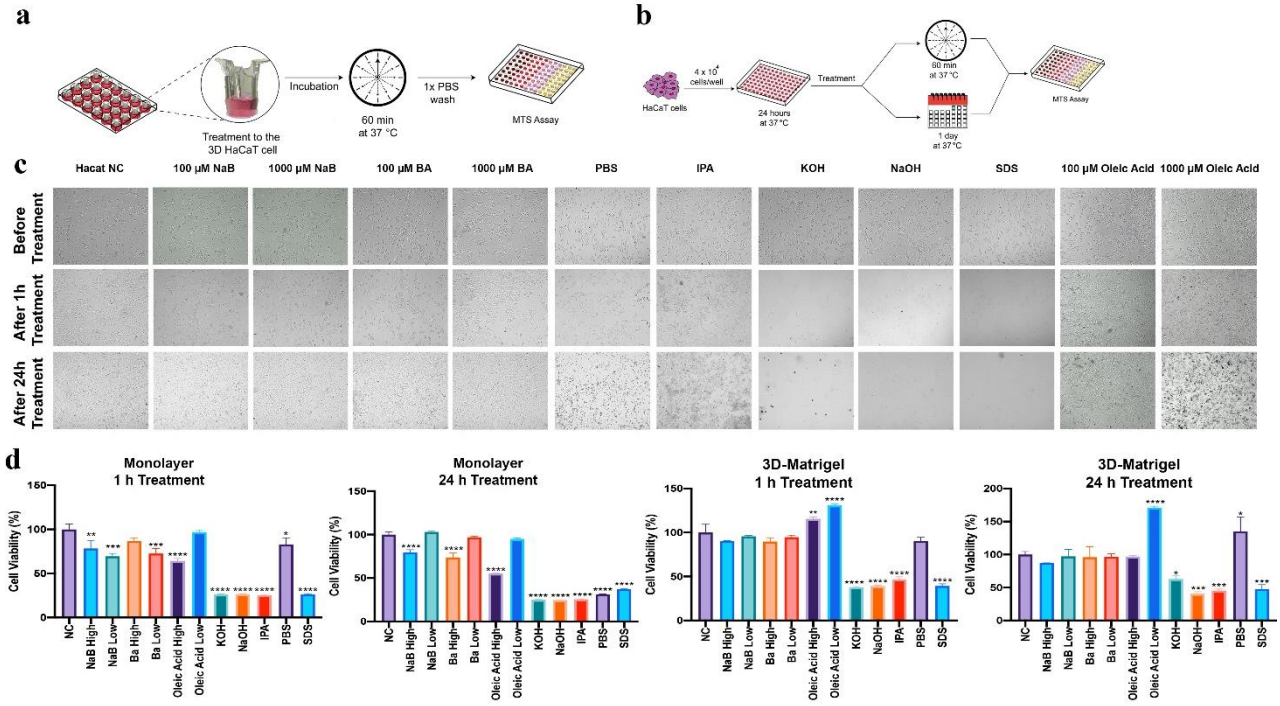

Fig. 3. Toxicology analysis of 2D and 3D HaCaT cell cultures. **a.** Experimental flow of toxicology analysis performed with 3D HaCaT cell cultures, **b.** experimental flow of toxicology analysis performed with 2D HaCaT cell cultures, **c.** light microscope images of toxicology analysis, **d.** cell viability analysis of 2D and 3D HaCaT cell cultures for 1 h and 24 h exposure time. NC: Cell culture media PC: 5% SDS *$p < 0.05$. PBS: Phosphate-buffered saline, 2-propanol: isopropyl alcohol, SDS: Sodium Dodecyl Sulphate, KOH: Potassium hydroxide, NaOH: Sodium hydroxide, BA: Boric Acid, NaB: Sodium pentaborate decahydrate.

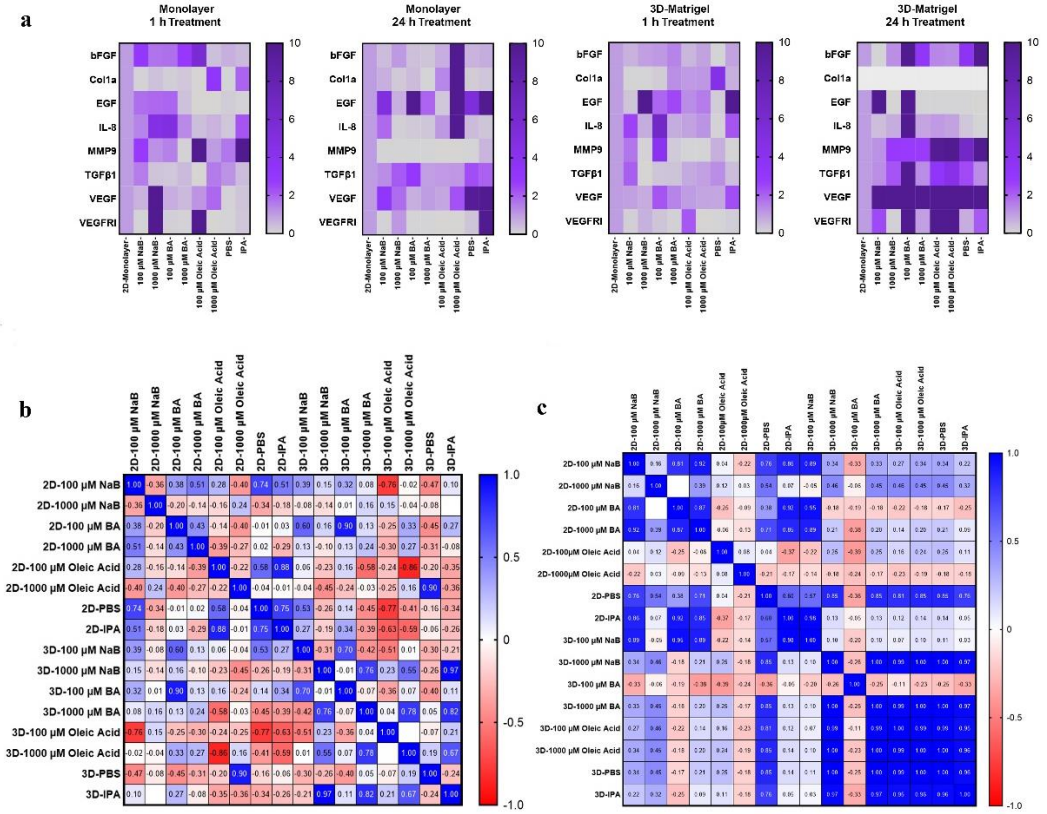

Fig. 4. qPCR analysis of 2D and 3D HaCaT cell cultures. **a.** Heat map representation of qPCR analysis, **b.** correlation matrix coefficient analysis of gene expression analysis for 1 h exposure time of 2D and 3D cell cultures, **c.** correlation matrix coefficient analysis of gene expression analysis for 24 h exposure time of 2D and 3D cell cultures. BA: Boric Acid, NaB: Sodium pentaborate decahydrate, PBS: Phosphate-buffered saline, IPA: isopropyl alcohol, bFGF: basic fibroblast growth factor, Col1A: Collagen-1A, EGF: Epidermal growth factor, IL-8: Interleukin-8, MMP9: Matrix metalloproteinase-9, TGFβ1: Transforming growth factor-β1, VEGF: Vascular endothelial growth factor, VEGFR1: Vascular endothelial growth factor receptor-1.

Discussion

Development of epidermis-like model systems is not only important for evaluating skin irritation but also for assessing cell viability, wound healing, and toxicity. Compared to primary cell cultures, HaCaT cultures have proven to be reliable sources of keratinocyte cells.

Inflammatory responses and cytokine releases vary when HaCaT cultures are treated with differing calcium concentrations, which suggests that they can be used as a model system for skin related pathologies.

In the current study, we created an extracellular matrix-based 3D culture using HaCaT cells which can be used as a reliable method for *in vitro* keratinocyte-based toxicity analysis. Although previous studies demonstrated that air-liquid interface exposures did not result in the complete epidermal differentiation of HaCaT cells, multilayered epithelium generation has been observed to some extent (Boelsma *et al.* 1999). Epidermal differentiation and barrier function were also observed in previously generated HaCaT and fibroblast based model (Schmidt *et al.* 2020). Monolayer keratinocyte cell cultures are not sufficient for evaluating cell viability or toxicity of chemicals which contact surface of skin. Therefore, the 3D HaCaT cell culture described here may be a valuable tool for *in vitro* toxicology analysis.

We replaced the standard fibroblast conditioned medium (do Nascimento Pedrosa *et al.* 2017) with an MEF conditioned medium. MEFs have been used for pluripotent stem cell cultures and secrete various factors to promote cell growth. We used the MEF conditioned medium as a growth factor and cytokine source to promote HaCaT cell proliferation and differentiation in culture. MEF conditioned medium contains various growth factors and cytokines such as RANTES, TIMP1, VEGF-A and VEGF-D. Furthermore, MEF cells are commercially available and can be used to obtain conditioned medium easily.

The three-dimensional HaCaT cell cultures showed a skin-like gene and protein expression profile indicating the similarity to actual epidermal structures. 3D HaCaT cell cultures expressed several skin related genes including bFGF, CoL1a1, EGF, FGF7, fibronectin, HB-EGF, IL-10, IL-8, MMP2, MMP9, TGFβ1, VEGF, VEGFRI, VEGFRII indicating the epidermis-like skin phenotype in culture. It has been shown in previous studies that fibronectin, TGFβ1 and VEGF are crucial for fibroblast-keratinocyte interactions and wound healing response (Gailit *et al.* 1994, Hamill *et al.* 2012, Larjava *et al.* 2013). Protein array analysis showed that 3D HaCaT cell cultures displayed an epidermis like protein expression pattern.

Standardized toxicity treatment of *in vitro* reconstituted skin generally varies between 15 and 60 minutes depending on the tested compound and its physical form (OECD, 2021). Cell viability analysis with monolayer culture systems use various time exposure periods which generally start with a 24 h analysis to observe a potential effect. However, dermal toxicology is a different application and toxicology analysis should mimic the actual skin exposure scenario. While KOH, IPA, NaOH and SDS are skin irritants, PBS is a non-irritant (Capallere *et al.* 2018, De Jong *et al.* 2020, Schmidt *et al.* 2020). Monolayer culture system-based toxicology analysis cannot mimic the actual skin structure and was found to be fragile in the current study even after 1 h PBS exposure which is normally not toxic for the skin. On the contrary, 1 h PBS exposure to the 3D HaCaT model did not, like living human skin, show any toxicity. Also, boron compounds which are not irritants to human skin (Jackson *et al.* 2020) or the 3D cultures, produced toxicity in the monolayer culture.

In the present study, we found that 3D HaCaT cell cultures generated with the Matrigel matrix displayed a skin-like response when dosed with selected test chemicals and results with positive and negative controls were appropriate. Culture media and SDS, the negative and positive controls, respectively, demonstrated similar gene expression profiles in the monolayer culture system, which was unacceptable. It has also been reported in that collagen serves as a support matrix for healthy skin and is a critical factor in the maintenance of skin firmness and suppleness. Type I collagen is the most abundant type found in the skin, accounting for between 80% and 90% of skin collagen. The CoL1a1 gene provides instructions for making the pro-α1(I) chain of type I collagen (Reilly & Lozano 2021). Here we demonstrated the increased amount of CoL1a1 protein by ELISA measurements. Based on these results, this 3D HaCaT model can be used as an alternative to *in vitro* test techniques including 2D cell culture models to evaluate the skin irritation potential of chemicals. It might be used as an alternative for RhE skin irritation models after improvements of limitations in our model. The advantage of our model compared to other 3D skin irritation models is that our model is less complex, less time consuming, and easier to adapt. However, we could not generate a fully layered skin like structure by using HaCaT cells and fibroblast conditioned medium. Mini *et al*. 2021 showed the epidermal stratification in their model using keratinocytes and fibroblast cells which lacked in our 3D model (Mini *et al.* 2021).

Currently, there are seven 3D RhE skin irritation models included in OECD Test Guideline No. 439 (OECD 2021). So, they can be used even for irritation assessment of medical devices (Kandarova *et al*. 2018, De Jong *et al.* 2020). These models, which were developed for testing neat chemicals, have been validated with a series of 20 irritant and non-irritant reference chemicals, and are recognized by OECD as standalone replacements for the rabbit skin irritation test.

The RhE models consist of multi-layered human epidermis differentiated from human-derived epidermal keratinocytes. Both polar and non-polar extracts can be tested on these models.

Two of the RhE models were evaluated in an international round robin study, which found that they could

detect low levels of irritant chemicals in dilute extracts of polymeric materials and medical devices (Kandarova *et al.* 2018, De Jong *et al.* 2020). Because our 3D model system did not generated a multi-layered human epidermis and lacks all cell types of the skin, it cannot be used to replace animal models and cannot be referred as a reconstructed skin.

According to this study, our 3D HaCaT skin model is suitable for determining cell viability and chemical toxicity. However, our model needs to undergo further testing and validation before it will be acceptable for medical use. Different skin cell types including fibroblast cells, immune cells, Langerhans cells and melanocytes should be included in the 3D model to mimic actual skin structure. Additional confirmation techniques including permeability should be conducted. Also, although we tested our model with various polar chemicals, non-polar extracts should also be tested. Still, considering the results, our 3D HaCaT skin irritation model has the potential for testing both neat chemicals and chemical mixtures.

In conclusion, our 3D HaCaT cell culture model could be a useful tool for testing the skin irritational potential of chemicals and an alternative research model. It is more straightforward and easy to establish compared to other models. Our model system is a more reliable alternative compared to monolayer culture systems and can be easily used for cell viability analysis in vitro. Although culture of HaCaT cells did not fully depict the epidermal differentiation, layered structures of the skin and cannot be used as an alternative to *in vivo* testing, 3D culture might be more reliable for *in vitro* toxicology analyses.

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