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Research Article

SALBUTAMOL AMELIORATES THE PHENOTYPE OF THE SKIN INFLAMMATORY DISEASE PSORIASIS ACCORDING TO SKIN SPHEROID MODELS

Özge Sezin SOMUNCU[1*], Berke DEMİRİZ[2], İrem TÜRKMEN[2], Salih SOMUNCU[3], Berna AKSOY[4]

[1]Stony Brook Medicine, Department of Pathology, New York, USA
[2]Bahçeşehir University School of Medicine, İstanbul, TURKEY
[3]Bezmialem Vakıf University Dragos Hospital, Department of Pediatric Surgery, İstanbul, TURKEY
[4]Bahçeşehir University Faculty of Medicine, Department of Dermatology, İstanbul, TURKEY

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Enes Taylan

****Corresponding Author:***
Özge Sezgin SOMUNCU
ozge.somuncu@stonybrook.edu

ORCID iDs of the authors:
ÖSS. orcid.org/0000-0002-0841-8263
BD. orcid.org/0000-0002-0419-1786
İT. orcid.org/0000-0003-1692-6417
SS. orcid.org/0000-0002-3154-5527
BA. orcid.org/0000-0003-2346-1865

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Abstract: Psoriasis is a multifactorial chronic inflammatory disorder resulting by the interplay of genetics, the immune system and the environment. It is characterized by the hyperproliferation of epithelial cells, generating red, itchy psoriatic plaques which have no cure but have great negative impact in patients' life. Although corticosteroids or vitamin D analogs might help recovery to some extent, there is yet no total cure for the disease. In this study, we sought to generate three-dimensional (3D) stress-related psoriatic skin spheroids with the screening of the potential efficacy of a β_2-adrenergic receptor agonist, salbutamol. 3D Culture spheroids with human dermal fibroblasts (HDF), human epithelial keratinocytes (HEK) and human monocytic cell line (THP-1) were generated as a representative model of skin and the protocol of stress-related modelling was conducted. The efficacy of the drug salbutamol was evaluated by the changes in mRNA and protein expression levels of selected genes, as well as by several metabolic assays. We developed a method for culturing spherical organoid models of psoriasis *in vitro*. We tested the potential theurapetic effects of salbutamol on psoriasis spheroids. Spheroids treated with salbutamol indicated the effictiveness of the treatment. 3D spheroid system was found partially efficient for mimicking the physiological features of psoriasis *in vitro*. This present work may be a starting point for future investigation as it is the first to generate a stress-related psoriatic model and first to try a β_2 agonist as a potential treatment option. Considering the effects and suitability of topical application of salbutamol, its efficacy should not be underestimated and should be investigated further for translating this knowledge into clinics.

Özet: Sedef hastalığı; genetik, bağışıklık sistemi ve çevrenin karşılıklı etkileşiminden kaynaklanan, çok faktörlü kronik inflamatuar bir hastalıktır. Epitel hücrelerinin hiperproliferasyonu ile karakterizedir ve hastaların yaşamında büyük olumsuz etkileri olan kırmızı, pullu psoriatik plaklar oluşturur. Kortikosteroidler veya D vitamini analogları iyileşmeye bir dereceye kadar yardımcı olabilse de hastalığın henüz tam bir tedavisi yoktur. Bu çalışmada, β_2-adrenerjik reseptör agonisti salbutamol'ün potansiyel etkinliğinin taranması için üç boyutlu (3D) stresle ilişkili psoriatik deri sferoidleri oluşturulması amaçlanmıştır. İnsan dermal fibroblast (HDF), İnsan epidermal keratinosit (HEK) ve İnsan monosit hücreleri (THP-1) ile 3D kültür modelleri oluşturulmuş ve buna göre stres kökenli psoriatik model protokolü uygulanmıştır. İlacın etkinliği, gen ve protein ekspresyon seviyelerindeki değişiklikler ve çeşitli metabolik deneylerle değerlendirilmiştir. Sedef hastalığının sferoid modellerini *in vitro* olarak büyütebilmek için optimize bir yöntem geliştirilmiştir. Salbutamol'ün sedef sferoidleri üzerindeki potansiyel terapatik etkileri test edilmiştir. Salbutamol ile tedavi edilen sferoidler, tedavinin etkinliğini kanıtlayan literatürle paralel sonuçlar göstermiştir. 3D sferoroid sistemimiz, *in vitro* olarak sedef hastalığının fizyolojik özelliklerini taklit etmede kısmen etkili bulunmuştur. Çalışmamız, stresle ilişkili bir psoriatik model oluşturduğu ve potansiyel bir tedavi seçeneği olarak bir β2 agonistini deneyen ilk çalışma olduğu için bir başlangıç noktası olabilir. Salbutamol'ün etkileri ve uygunluğu göz önünde bulundurulduğunda etkinliği küçümsenmemeli ve gelecekte klinikte kullanım potansiyeli göz önünde bulundurulmalıdır.

Introduction

Psoriasis is a chronic inflammatory disease that affects around 125 million people, in other words 2-3% of human population in the world. It is triggered by multifactorial interactions among the immune system, psoriasis-related susceptibility loci (*PSORS1*), auto-antigens, and several environmental triggers (Takeshita *et al.* 2017). The stimulation and upregulation of IL-17 in pre-psoriatic skin creates an inflammatory reaction in keratinocytes that forms the expansion of advanced psoriatic plaques by enhancing epidermal hyperplasia, epidermal cell proliferation, and recruitment of leukocyte branches into the skin (Hawkes *et al.* 2017).

Conventional therapies for psoriasis are typically topical therapies which mostly end up with possible severe side effects. Topical therapies include keratolytics, topical retinoids, topical vitamin analogs, and calcineurin inhibitors. While topical corticosteroids remain first-line treatment that aid alleviating all grades of psoriasis, unwanted side effects including atrophy, striae and/or telangiectases contraindicates their long-term utilization (Torsekar & Gautam 2017). The dual use of corticosteroids and vitamin D analogs display greater efficiency as compared to monotherapy; but side effects like skin irritation, erythema and edema are shown in up to 35% of the patients (Sharma *et al.* 2017). Although more recently developed biological agents such as TNF antagonists, specific monoclonal antibodies, phosphodiesterase 4 or phospholipase A2 inhibitors offer improved anti-psoriatic therapeutic responses, they also pose risk of adverse effects, are expensive, and the potential for development of tolerance or resistance may limit their use (Sharma *et al.* 2017). Hence, there is a need to develop new cost-effective therapies with low side effects.

Commercially accessable psoriasis models are composed of healthy keratinocytes and unhealthy fibroblasts which are isolated from psoriatic lesions of patients. Van den Bogaard *et al.* (2014) were the earliest to complete the generation of three-dimensional (3D) skin counterparts that included diverse T-cell populations. Their study enabled the analysis and relocation of immune cells and discharge of pro-inflammatory cytokines in the context of psoriasis. Nevertheless, hyperproliferation was not detected in 3D skin and cytokine levels were much lower compared to the *in vivo* generated lesion, signifying that in 3D models, critical constituents and pertinent cell types were absent to generate a more accurate psoriasis model (Klicks *et al.* 2017). Up to now, only a few organotypic models emphasize the importance of different cell types in psoriasis, therefore it is important to investigate the inflammatory microenvironment of multi-cellular psoriatic *in vitro* models (Eline Desmet *et al.* 2017). Since animal models cannot reflect the human complexity for the multifactorial etiology of psoriasis, generation of an optimal 3D psoriasis model made by human cells remains crucial (Eline Desmet *et al.* 2017).

Salbutamol is a well-known β_2-adrenergic receptor (β-AR) agonist in the treatment of asthma as well as chronic obstructive pulmonary disease. The inhibitory effects of salbutamol on inflammatory processes is seen for CD4+ cells, monocytes and macrophages and it acts through the inhibition of the ERK pathway (Keränen *et al.* 2017). In addition, anti-inflammatory effects of β-AR on pulmonary inflammation models support the role of receptors in inflammatory conditions (Bosmann *et al.* 2012). There are a couple of studies in literature investigating the effects of salbutamol on psoriasis. Wettey *et al.* (2006) showed the inhibitory effect of salbutamol on CXCR2 (C-X-C Motif Chemokine Ligand 2) which is elevated in psoriatic lesions. A recent study showed the ameliorating effect of salbutamol on psoriasis, correlating with our studies (Liu *et al.* 2020).

Psoriasis has been associated with wound healing and one recent study indicated that in murine skin wound models, stress-induced increase in epinephrine levels were found to delay wound repair (Pullar & Isseroff 2006). Filaggrin 2 is essential for healthy cornification of skin and it functions in skin barrier defense. The expression of Filaggrin 2 was found to be reduced in psoriasis vulgaris in previous studies (T. Makino *et al.* 2014). Matrix metalloproteinase-2 (MMP-2) cleaves native collagen type IV, V, VII, and X, fibronectin, osteonectin, entaxin, laminin, vitronectin, decorin, gelatin, and aggrecan, several chemokines (CCL7 and CXCL12), Tumor Necrosis Factor (TNF) precursors and proTNFβ (Starodubtseva *et al.* 2011). Previous studies showed significant overexpression of MMP-2 in psoriatic skin (Glazewska *et al.* 2016). Interleukin 6 (IL-6) produced from keratinocytes has been shown to be responsible for the inflammation in psoriatic skin lesions (Fujishima *et al.* 2010). Recently, it has been shown that fibroblasts produce IL-8 in cell culture while higher concentrations of IL-8 was detected in psoriatic patients (Glowacka *et al.* 2010). Filaggrin-2, MMP-2, IL-6 and IL-8 were selected as markers in our study depending on their involvememt to psoriasis disease progression. Additionally, an elevated total oxidant status and inadequate antioxidant activity have been defined in psoriatic lesions. The endogenous antioxidant defence mechanism of the body is insufficient to replenish the impairment, and the inadequate skin metabolism deteriorates the state of the skin in psoriasis patients (Asha *et al.* 2017).

In this study, we aimed to generate an optimized 3D stress-related psoriatic skin model along with the investigation of the potential theraupetic effect of salbutamol, a β_2-adrenergic receptor agonist in this psoriatic spheroid model.

Materials and Methods

Cell Culture

Human dermal fibroblasts (HDF) and human epithelial keratinocytes (HEK) were purchased from American Type Culture Collection (ATCC, USA) that was isolated from the newborn foreskin (prepuce) tissue. Briefly, cells were plated in 6-well plates (BIOFIL, TCP,

Switzerland) and grown until 80% confluency in low Dulbecco's Modified Eagle Medium (DMEM) (Gibco/Invitrogen) media supplemented with 10% (v/v) heat-inactivated Fetal Bovine Serum (FBS) and 1% penicillin/streptomycin solution for human dermal fibroblasts according to the reference study (Somuncu *et al.* 2015) and in Defined Keratinocyte -SFM (Serum Free Medium) supplemented with Keratinocyte Growth Supplement (Sigma Aldrich, Germany) and 1% penicillin/streptomycin solution for human epithelial keratinocytes. For passaging, the cells were trypsinized using 0.25% (v/v) trypsin/EDTA (Invitrogen, Gibco, UK) and centrifuged at 1200 rpm for 5 min at room temperature in order to precipitate cells. The pellets were then resuspended in fresh medium accordingly and seeded into T-75 flasks (Zelkultur Flaschen, Switzerland) containing 10 ml media. The cells were preserved at 37°C and 5% CO_2 in a humidified incubator. Cells from passages 3 ~ 4 were used for experiments. THP-1 (Human Monocytic Cell Line) and Human Dermal Microvascular Endothelial Cells (HDMEC) were purchased from American Type Culture Collection (ATCC, USA) and cultures were established following to centrifugation and resuspension at 2×10^4 viable cells/ml. The heterogenous psoriatic cell population (PsorI) induced from keratinocyte cell line by defined protocol (E. Desmet *et al.* 2017) was used as a positive control during the study.

Generation of Skin Spheroids

After removing the media and washing the cells with 1 mL Dulbecco's phosphate-buffered saline (D-PBS) without calcium and magnesium, cells were trypsinized and resuspended in Matrigel as 2×10^5 cells/ml density. Matrigel droplets including HDF, HEK and HDMEC cells were added as 50 µl bubbles into each insert of a Transwell plate (Life Technologies, CA, USA) and incubated for 5-7 days in 1:1 diliution of DMEM and Keratinocyte SFM-1X (ThermoFischer, Turkey) media. Subsequently, 5×10^5 THP-1 monocytes were added to the bottom chamber, cultured for 2 days more and the medium was changed in every three days in top well. Then, the medium was only added to the lower chamber of the insert to generate an air-liquid interface. Spheroid constructs were incubated in Orbital Shaker-Incubator (bioSan, UK) at 37 °C and 5 % CO_2 for 21 days (Vörsmann *et al.* 2013) (Fig. 1a).

Modeling Stress-Related Psoriatic Skin Spheroids

Healthy spheroids were further utilized for disease modeling on 21st day of the procedure. Firstly, UV application was performed for 5 minutes in every two days of one week (Weatherhead *et al.* 2011). At the end of the first week of the protocol, fresh media containing IL-17 was applied and spheroids were incubated for another one week (Chiricozzi *et al.* 2014). At the end of the second week, the media was refreshed and macrophage-activating factor (MAF) administration was performed for 3 days to alert immune cells (Takematsu & Tagami 1990) (Fig. 1a). Samples were incubated at 37°C and 5% CO_2 in Orbital Shaker-Incubator (bioSan, UK). The timeline was established as day 7 (week 1) spheroids, day 14 (week 2 spheroids) and day 40 (cells of spheroids that reseeded in monolayer environment) (Fig. 1b).

Microscopical Analysis of Spheroids

Spheroids were visualized after UV treatment, MAF application, IL-17 application and combination of MAF and IL-17 application with UV treatment in week 1 and week 2 by bright-field microscopy. After optimization of psoriasis modeling, psoriatic skin spheroids were left for incubation and they were visualized in day 7, day 14, day 21 and day 40. Visaualization was accomplished in 40× magnification by ZEISS inverted microscope (Ivascu & Kubbies 2006).

Cell Viability Assay

Salbutamol (S8260-50MG) was purchased from Sigma-Aldrich and used for drug toxicity analysis of heterogenous population of HDF and HEK cells and THP-1 cells. Salbutamol was dissolved in High Glucose DMEM and administered to the cells from 0 to 4.4 µg with 0.2 µg intervals. 3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyltetra-zolium bromide (MTT) cell viability analysis was done after the drug application. Cells were plated in 96 well plates with 5,000 cells per well and incubated for 24 hours. After 24 hours of incubation, salbutamol was applied, and the cell viability was determined for day 1 and day 3. MTT reagent was adminstred as 10 µl to 90 µl of cells and media mix and incubated for 3 hours until purple precipitate was visible. Then, 100 µl Detergent Reagent was added and incubated at room temperature in dark for 2 hours. Absorbance of MTT was recorded at OD 570 nm (Bahuguna *et al.* 2017).

Immunofluorescence Analysis

Cryomolds were organized for Immunofluorescence staining. Optimal Cutting Temperature (OCT) compound was put into plastic cryomolds. Spheroids were positioned on top in correct orientation and OCT was applied by avoiding bubbles until none of the tissue remains uncovered. Mold was placed on top of the aluminium plate on dry ice for rapid freezing. Frozen sections were cut as 8 µm sections and mounted onto slides. Slides were washed with PBS for three times for 5 minutes. Every tissue section was marked with hydrophobic pen (Imedge Pen). Slides were blocked with Blocking Buffer (PBS with 5% horse serum and 0.5% Triton X-100) at room temperature for 1 hour. Slides were then incubated with primary antibodies; Anti-Filaggrin Antibody (ab218395) (1 µg/ml), Anti-Cytokeratin 15 Antibody (ab80522) (5 µg/ml), Anti-IL6 Antibody (ab9324) (1 µg/ml), Anti-IL8 Antibody (ab18672) (1 µg/ml) diluted in blocking buffer at 4°C overnight. Slides were washed with PBS for three times for 5 minutes subsequently. Incubation with secondary antibody Alexa Fluor® 647 Goat Anti-Mouse Antibody (ab150115) (1:200 dilution) was done at room temperature for 1 hour. PBS was used for washing the slides. Slides were stained with DAPI for 1 minute and

fixed with mounting solution. Imaging was performed on Leica DMLB Phase Contrast Fluorescence Microscopy. Fluorescent images were merged with ImageJ software (Ö. S. Somuncu *et al.* 2019).

Quantitative Real Time Analysis for the Detection of Gene Expression

The samples were grouped as healthy skin spheroids, heterogenous PsorI cell population, UV treated spheroids, UV and IL-17 treated psoriasis spheroids, psoriasis spheroids after 10 h salbutamol treatment and psoriasis spheroids after 24 h treatment. According to the instructions, isolation of RNA from each group of samples was done by using High Pure RNA isolation Kit (Roche, Germany). The complementary DNA (cDNA) synthesis from isolated RNA templates was provided with High Fidelity cDNA Synthesis Kit (Roche, Germany). Real time polymerase chain reaction (qPCR) was performed by using Maxima SYBR Green/ROX (Fermentas, US) to determine expression levels of target genes that comprises Keratin 1, Filaggrin 2, IL-6 and MMP-2. The cDNA templates were utilized and mixed with primers and Maxima SYBR Green/ROX qPCR Master Mix (2×). Glyceraldehyde 3-Phosphate Dehydrogenase (GAPDH) was used as house-keeping gene for data normalization (S. Somuncu *et al.* 2019). The results of real-time PCR were obtained via performing normalization with GAPDH. Primer sequences for target genes are shown in Table 1.

Table 1. Primers designed for detection of *MMP-2*, *IL-17*, *Keratin 1*, *Filaggrin 2*, and *IL-6* expression.

Names of the Genes	Primer Sequence (5'-3')
MMP2	Forward Primer: AGCGAGTGGATGCCGCCTTTAA Reverse Primer: CATTCCAGGCATCTGCGATGAG
Filaggrin 2	Forward Primer: CCACACTCACGAGAACACA Reverse Primer: ACCAGAGTGGGAATGTCCAG
Keratin 1	Forward Primer: AGGGTTGTAGGAGCCTTGAC Reverse Primer: CCACTCCAGTGAGGCCAATA
IL-6	Forward Primer: GGGGCTGCCTGCATTAGGAG Reverse Primer: AAGCCCGGGGGACAAAAAGG

All primers were designed by our group. The primers designed with the annealing temperature 60C°.

Total Antioxidant and Oxidant Assay

Total Oxidant Status (TOS) and Total Antioxidant Status (TAS) of each experimental group were measured according to instructions. Sample media was stored at -80°C for the analysis of TAS and TOS. Total Antioxidant Status Assay Kit (Sigma-Aldrich) was used and the kit protocol was followed for TAS determination. 1 mM Trolax standard solution was used for creating a standard curve by setting up different dilutions. 100 µl of Cu^{2+} working solution was added to each well containing standard and samples. Wells were then mixed and incubated at room temperature for 90 minutes. The plate was then transferred to a microplate reader to be analyzed at OD 570 nm (Miller *et al.* 1993). TOS was examined by Erel's TOS method that is about the oxidation of ferrous ion to ferric ion in the existence of diverse oxidative species in the acidic medium. Ferric ion was analyzed by xylenol orange. Briefly, xylenol orange, NaCl and glycerol in a H_2SO_4 solution were incubated with samples for 3 minutes. Ferrous ion and o-dianisidine in H_2SO_4 were applied to the reaction subsequently. The alteration in absorbance was examined, and the results were analyzed by a standard curve of H_2O_2 solution and expressed in µmol/L (Erel 2005).

Statistical Analysis

Complete data sets were presented as means ± standard errors (SEM). Graphics were drawn via GraphPad Prism 8 software (GraphPad Prism, USA). The statistical inquiry of the grades was completed by using one-way ANOVA trailed by multiple-comparison Tukey's Post-Hoc tests with GraphPad Prism 8 software. The stars were stated to flag levels of significance. A p-value less than 0.05 was considered statistically significant (Alabi *et al.* 2019). Heat maps demonstrating gene expression by quantitative real-time PCR were clustered in complete linkage of Heatmapper software presenting both column and row dendogram of hierarchical clustering (Babicki *et al.* 2016). Row Z-scores were demonstrated as green for high values and red for low values, respectively.

Results

UV, IL-17 and GC-MAF sequencial application generated psoriasis-like spheroids

For the optimization of psoriasis modeling, the microscopic phenotypes of the cells after variable UV, IL-17 and MAF treatments were compared with heterogenous psoriatic cell population at the end of week 1 and week 2. With UV treatment to healthy skin spheroids, small sized, dispersed and multiple spheroids were observed in week 1 and at the end of the week 2. The sizes of the spheroids were bigger, total number was increased and they were scattered over the surface instead of generating clusters. After MAF application to skin spheroids, bigger clusters were observed in increased numbers in week 1 and a complete cluster resembling the original lesion was visualized after week 2. When MAF application was combined with UV treatment, these clusters tended to seperate and dissolved completely at the end of week 2. With only IL-17 application, the clusters were bigger in size but more seperate on the surface, resembling seperate spheroidic islands in week 1, and the number of these bigger clusters were decreased with increased number of tiny additional spheroids between them. Combinational UV treatment with IL-17 induced separate clusters to disappear and bigger singular spheroids to appear in week 1, with a dramatic decrement

in quantity while the isolated spheroids were detected in increased size at the end of week 2. The protocol was optimized as UV treatment for 5 minutes in every two days of one week, IL-17 application for one week after UV treatment, and 3 days of GC-MAF application. After optimization of psoriasis modeling protocol, generated psoriasis spheroids were visualized in day 7, day 14 and day 40. Day 7 and day 14 samples indicated the differences in spheroids while day 40 samples were analyzed as monolayer cells of the spheroid content. Spheroids generated clusters, in time resembling a complete psoriatic skin lesion that was established by the monolayer phenotype of cells at the end of day 40 (Fig. 1b).

Cell viability for both THP-1 and HDF cells were established

In order to determine the highest toxic level of salbutamol on HDF, HEK, and THP-1 cells, 22 different concentrations of salbutamol were employed. After dose-dependent drug application, cell viability assay was performed at day 1 and day 3 to establish the optimal dose of drug for further experiments. The highest non-toxic dose for HDF cells was found as 1.6 µg/ml for both day 1 and day 3. For THP-1 cells, despite the peak seen in 2 µg/ml, the optimal dose was determined as 0.8 µg/ml due to the consistency of results in day 1 and day 3 (Fig. 1c).

Salbutamol treatment rescued the psoriasis-like gene expression profile

With the quantitative analysis of PCR (qPCR), relative mRNA expression levels of Keratin 1, Filaggrin 2, IL-6 and MMP-2 were determined before modeling, after modeling and after treatment. Relative Keratin 1 expression was increased with UV application but fell by half with the completion of disease modeling, resembling the levels of psoriatic cells. After salbutamol treatment, no significant change was observed in Keratin 1 expression. Similarly, Filaggrin 2 expression was increased two-fold after UV treatment and decreased three times after completion of modeling, resembling the levels of psoriatic cells. At the end of the treatment with salbutamol, Filaggrin 2 expression levels showed two-fold increase compared with disease model. Likewise, IL-6 expression was increased two-fold with UV application but decreased following the completion of modeling, similar to the levels of psoriatic cells. In the first 10 hours, salbutamol treatment caused a two-fold decreased expression of IL-6 with a subsequent five-fold increment at the end of the treatment. Distinctly, MMP-2 expression was decreased almost three-fold with disease modeling and increased four-fold with 10 hours of salbutamol treatment. At the end of the drug application, expression levels were close to disease models. While Keratin 1, Filaggrin 2 and MMP-2 showed decreased expression after disease modeling, no significant change was observed in IL-6 levels. Salbutamol treatment caused significantly increased expression of Keratin 1, Filaggrin 2, MMP-2 and IL-6 levels when compared between the treatment and psoriasis organoid groups (Fig. 2).

MMP-2 and IL-6 showed similar expression patterns in psoriasis-like spheroids

In order to visualize relative differences in mRNA expression of healthy skin spheroids, Psor1 cells, UV treated spheroids, UV and IL-17 treated complete psoriasis spheroids, psoriasis spheroids after 10 h salbutamol treatment and psoriasis spheroids after salbutamol treatment completed, a heat map was drawn. During modeling, UV treatment caused a decrease in gene expression levels of MMP-2, IL-6 and Filagrin 2 but an increase in the levels of Keratin 1. After modeling, MMP-2, Keratin 1 and Filagrin 2 showed a decreased gene expression, whereas IL-6 levels were increased compared to healthy skin spheroids. Filaggrin 2 and Keratin 1 expressions in psoriasis model showed high resemblance to lesion, whereas MMP-2 and IL-6 expression levels of psoriasis models were the opposite of lesion. After 10 hours of treatment with salbutamol, IL-6, Keratin 1 and Filaggrin 2 expression levels were decreased but MMP-2 levels were increased significantly. At the end of the treatment with salbutamol in psoriasis spheroid model, MMP-2 Filaggrin 2 and IL-6 shared a similar enhanced gene expression pattern and Keratin 1 showed a diminished expression after treatment. Hierarchical clustering of each gene group showed that Keratin 1 and Filaggrin 2 expression indicated the maximum gene expression correlation and the most irrelevant genes were detected as MMP-2 and IL-6 (Fig. 2).

Salbutamol treatment decreased cytokine expression but increased the expression of filament associated protein

Given that Cytokeratin 15, Filaggrin 2, IL-6, IL-8 and IL-17 expression is crucial in the pathophysiology of psoriasis, the expression of each of these was determined with fluorescent IHC in healthy skin model, psoriasis model and after the treatment with salbutamol in psoriasis spheroid model. Cytokeratin 15 expression was increased almost three-fold after disease modeling, then decreased into one fourth of the expression level of disease model after salbutamol treatment, correlating with Keratin 1 mRNA expression. Filaggrin 2 expression was also increased almost three-fold with disease modeling and although less significant, a decrement was seen in the expression after salbutamol treatment. IL-17 expression pattern was observed highest in healthy skin spheroid and decreased both with disease modeling and after treatment, for which expression levels were one fifth of the healthy one. After salbutamol treatment, IL-6 levels decreased into half and IL-8 levels were also decreased, although less significant. Cytokeratin 15, Filaggrin 2, IL-17, IL-6 and IL-8 all shared a similar pattern which was decreased expression after salbutamol treatment. While Cytokeratin 15 and Filaggrin 2 expressions were increased significantly with psoriasis models, IL-17 levels were decreased after modeling in a less significant manner (Fig. 2).

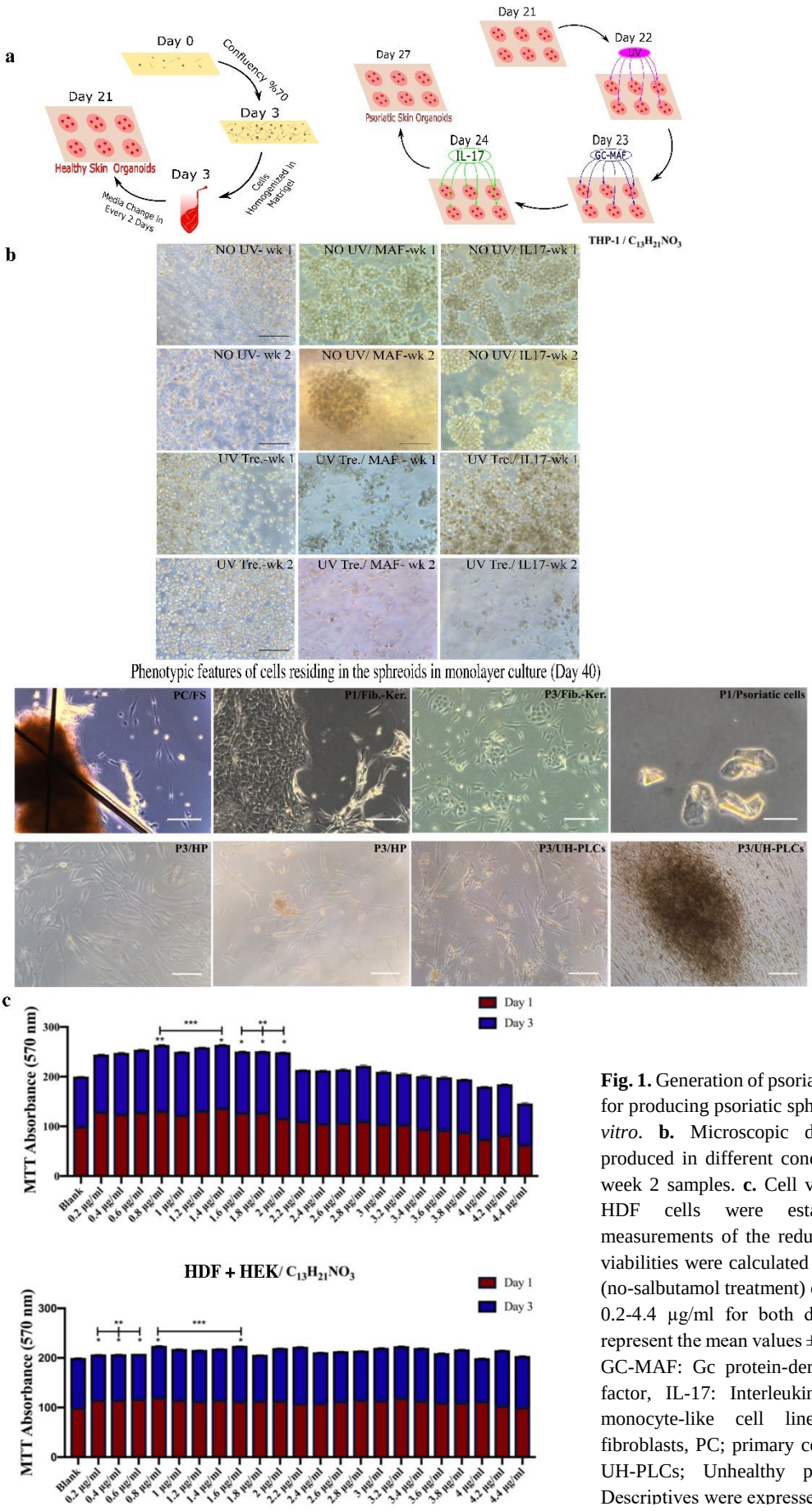

Fig. 1. Generation of psoriatic skin spheroids. **a.** Method for producing psoriatic spheroids in 27 days duration *in vitro*. **b.** Microscopic demonstration of spheroids produced in different conditions for both week 1 and week 2 samples. **c.** Cell viability for both THP-1 and HDF cells were established by colorimetric measurements of the reduction product of MTT. Cell viabilities were calculated as a percentage of the blank (no-salbutamol treatment) over a concentration range of 0.2-4.4 µg/ml for both day 1 and day 3. The data represent the mean values ± SD (n = 3). UV: Ultraviolet, GC-MAF: Gc protein-derived macrophage activating factor, IL-17: Interleukin-17, THP-1; Immortalized monocyte-like cell line, HDF; Human dermal fibroblasts, PC; primary cells, HP; healthy population, UH-PLCs; Unhealthy psoriasis like cells. Note: Descriptives were expressed as mean±standard error.

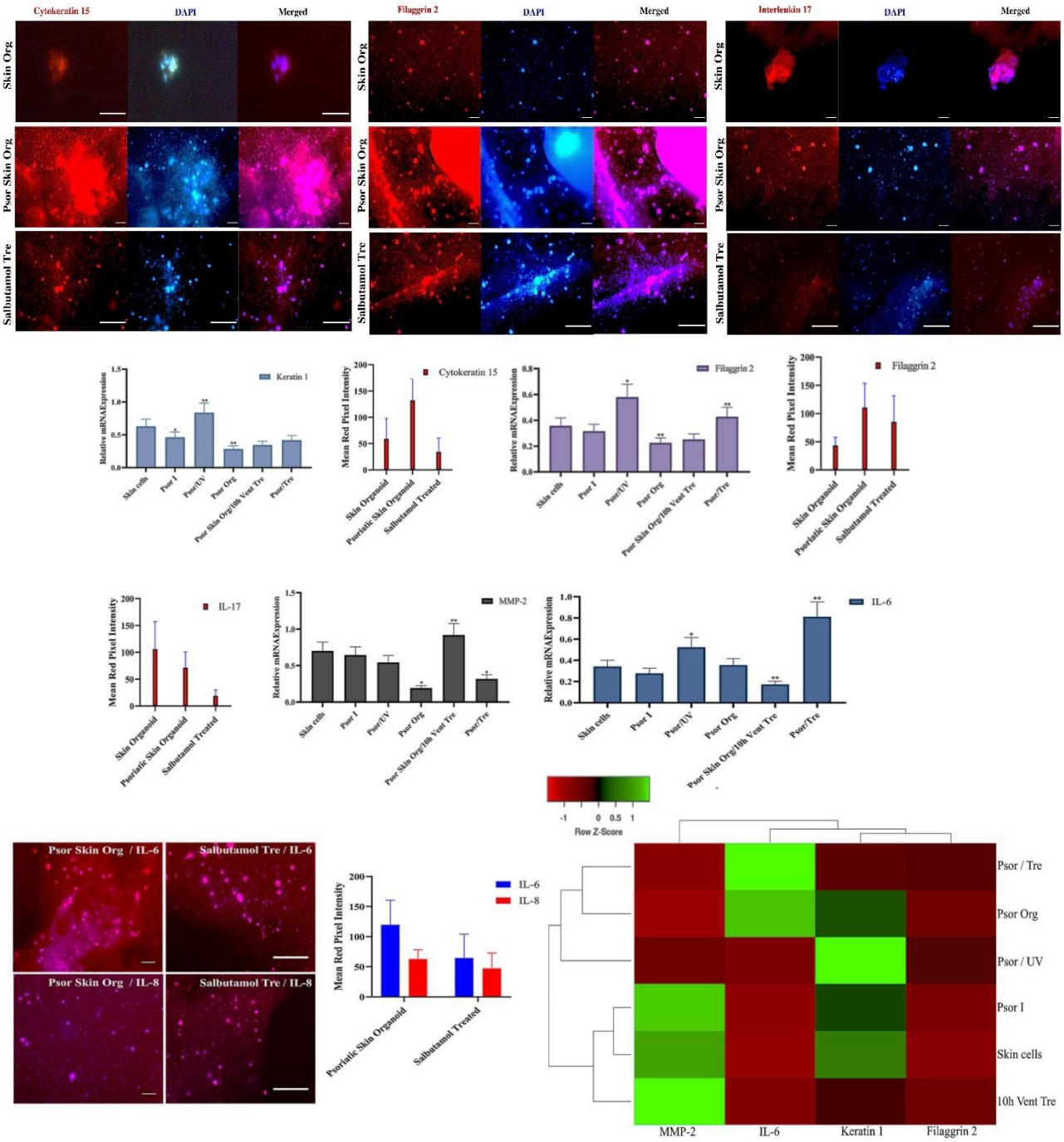

Fig. 2. Gene and protein expression profile of skin and psoriasis spheroids combined with salbutamol treated spheroids in each group. Heat Map presenting the gene expression data for a range of genes analyzed by qPCR. Quantitative representation of genes analyzed by quantitative RT-PCR and proteins analyzed by immunofluorescent staining. Experimental groups are established as following; Psor/Tre: Psoriatic spheroids/organoids treated with salbutamol for 24 hours, Psor Org: Psoriatic organoids, Psor/UV: Psoriatic spheroids induced with UV, Psor I: Psoriatic cells, 10h Vent Tre: Psoriatic spheroids treated with salbutamol for 10 hours. MMP-2: matrix metalloproteinase-2, IL-17: Interleukin-17, IL-6: Interleukin-6, IL-8: Interleukin-8, qPCR: Quantitative real time polymerase chain reaction. Statistically significant at $p<0.05$. Notes: Results were examined by one-way ANOVA and Tukey's Post-Hoc test. Descriptives expressed as mean±standar error.

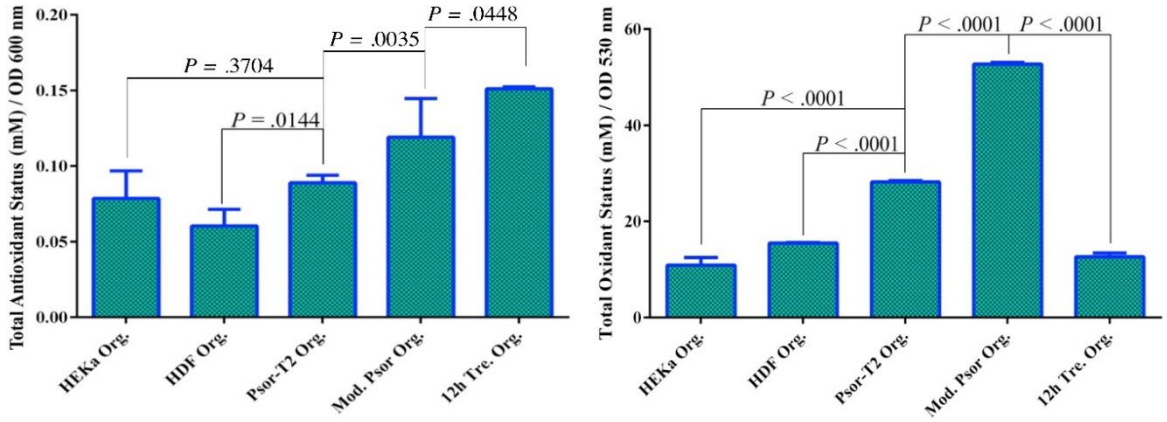

Fig. 3. TAS and TOS levels of the media obtained from treated and untreated spheroid cultures. Experimental groups are established as following; HEKa: Adult normal human epidermal keratinocytes, HDF org: Organoids/spheroids created with human dermal fibroblasts, Psor-T2 Org.: Organoids/spheroids created with Psoriasis type 2 cells, Mod. Psor. Org.: Modelled psoriatic organoids, 12h Tre. Org.: Modelled psoriatic organoids treated with salbutamol for 12 hours. OD: optical density, TAS: total antioxidant status, TOS: total oxidant status. Statistically significant at $p<0.05$. Notes: Results were examined by one-way ANOVA and Tukey's Post-Hoc test. Descriptives expressed as mean±standar error.

Salbutamol treatment significantly decreased oxidant production of psoriatic spheroids

Total oxidant and total antioxidant levels were measured from the cell culture media where the spheroids were grown. After modeling, a five-fold increment ($p<0.0001$) in total oxidant levels were measured and this increment was observed two-fold in psoriatic skin lesion. After 10 hours of treatment, total oxidant levels cut into half ($p<0.0001$) and at the end of the treatment, it was observed one fourth of the levels of psoriasis spheroids ($p<0.0001$). After modeling, the total antioxidant levels were increased significantly ($p=0.0144$). Although not significant, total antioxidant levels were higher in psoriasis model and after 10 hours treatment, two-fold augmentation of total antioxidant levels were observed with still a significant increment after salbutamol treatment (Fig. 3).

Discussion

In this study, we developed a method for culturing spherical organoid models of psoriasis *in vitro*. We tested the potential theurapetic effects of salbutamol, a β-AR agonist, on psoriasis spheroids. Our hypothesis was grounded in the anti-inflammatory effect of salbutamol, which was assumed to act through inhibition of cAMP dependent ERK pathway, thus causing decreased TNF-alpha and MCP-1 production in macrophages. Clinical observations suggested that systemic β-adrenergic blockade may trigger the onset of psoriasis-like skin lesions in some patients (Balak & Hajdarbegovic 2017) (Steinkraus *et al.* 1993). It was reported that the hyperproliferation of keratinocytes in psoriasis was the result of a decreased cAMP production intracellularly, thus current treatment options such as glucocorticoid treatment, UVB irradiation or topical vitamin D treatment have been shown to act through generation of cAMP in response to β-AR in keratinocytes (Sivamani *et al.* 2007). Although a known mechanism was reported before, none of the β_2-agonist drugs were tested for their efficacy in psoriasis patients. Therefore, this study aimed to investigate the efficacy of a β_2-agonist drug in a 3D psoriasis spheroid model for the first time.

Optimization of our psoriasis modeling protocol was determined based on the resemblance of the phenotypes of the spheroids to psoriatic cells upon the application of different stress factors. While UV treatment was observed to cause dysmorphism in the shape of the spheroids, MAF and IL-17 applications resulted in generation of clusters resembling psoriatic skin lesions. Combination of UV treatment with MAF and IL-17 caused dysmorphic clusters and prolongation of this treatment resulted in the loss of colonies. After optimization and incubation of 40 days, psoriatic spheroids generated tight clusters resembling a real psoriatic skin lesion.

Cell viability assays indicated that none of the dosage of salbutamol was toxic. After examination of each dose, 0.8 μg/ml, 1.6 μg/ml and 3.2 μg/ml were selected and evaluated as they were drawn attention for different cell lines. At the end, the optimal dosage was selected as 1.6 μg/ml for application of the drug. As none of the dose was found toxic, this gave the idea of salbutamol being physically processed on skin cells and effective on their cellular behavior.

Psoriatic epidermis is known to have decreased Keratin 1(K1) and K10 levels which are differentiation specific keratins and increased K6 and K16 levels (Thewes *et al.* 1991). In one study, it was suggested that Keratin 15 expression resulting from resident proliferating keratinocytes in the basal layer was uniquely downregulated in hyperproliferative situations such as psoriasis to maintain the activated phenotype of keratinocytes (Waseem *et al.* 1999). Our results showed an increased level of K1 after UV treatment, and a subsequent decrease in these levels were observed at the end of the modeling process suggesting that IL-17 and G-CSF might interfere with Keratin 1 expression in

psoriasis. On the other hand, the protein expression level of Keratin 15 was increased significantly with disease modeling and after treatment, it decreased to even lower levels than the beginning. Since no detailed study was found in the literature suggesting Keratin 15 differences, the mechanism behind this regulation should be further investigated.

Psoriasis is thought to to have a complex autoimmune and inflammatory pathophysiology with a genetic basis. It is thought that IL-17 induced release of keratinocyte-derived inflammatory mediators from TNF pathway form the key mechanisms driving psoriasis pathogenesis and it was seen that those two pathways are affecting each other (Ogawa *et al.* 2018). Supporting this idea, Fujishima et al. (2010) demonstrated that IL-17 stimulates the production of IL-6 from keratinocytes and is responsible for the inflammation in psoriatic skin lesions. Thus, current medical treatments and new small molecules comprising immunotherapies try to decrease the levels of those cytokines. In our study, IL-17 application was used to optimize the psoriasis modeling. Given the result of decrement after salbutamol treatment, it is concluded that psoriasis spheroid model reflects the disease and respond the therapy. IL-6 mRNA and protein expression also increased with disease modeling but decreased significantly after treatment. Despite the decreased protein expression at the end of the therapy, significantly high IL-6 mRNA levels were found, suggesting a different factor interfering with the translation mechanism. IL-17 and IL-6 pathways are known to upregulate IL-8 levels in keratinocytes, which leads to microabscess formation by enhancing neutrophil recruitment in psoriasis (Ogawa *et al.* 2018). Although IL-8 was not significantly changed, IL-6 which is a master regulator of both inflammation and metabolism (Ghanemi & St-Arnand 2018) significantly decreased after treatment.

IL-17 also seemed to be responsible for the reduction of Filaggrin 2 levels in psoriatic lesions and this alters the differentiation of keratinocytes as a part of the pathophysiogical mechanism (Gutowska-Owsiak *et al.* 2012) (Teruhiko Makino *et al.* 2014). Consistently, an increase in Filaggrin 2 protein levels were seen with a decreased IL-17 levels with disease modeling. Although Filaggrin 2 protein expression seemed to increase with disease modeling, mRNA levels first increased with UV treatment but decreased significantly after IL-17 and MAF application, which correlates with the literature (Simonsen *et al.* 2017) (Gutowska-Owsiak *et al.* 2012). After salbutamol treatment, an increased mRNA level of Filaggrin 2 was observed in spite of a decreased level of Filaggrin 2 protein expression. mRNA levels were more consistent with literature data due to decreased levels with disease modeling and increased levels after treatment.

MMP-2 stimulation by IL-6, IL-17 and various other inflammatory cytokines has been reported to be crucial in early progression of psoriasis (Jovanovic *et al.* 2000) (Sun *et al.* 2014). The key role of this molecule is in the modification of ECM and basement membrane, as well as cell migration and tissue remodeling activation. Feliciani *et al.* (1997) was the first to report the significant overexpression of MMP-2 in psoriatic skin. Conversely, our results indicated significantly decreased levels of MMP-2 with disease modeling, and an obvious increase with the salbutamol treatment in the first 10 hours, with a return to the starting levels at the end of the treatment. Application of IL-17 or MAF during disease modeling might be the reason for the MMP-2 expression.

Since psoriasis was a state of oxidative stress, enhanced total oxidant levels and decreased total antioxidant levels were reported in psoriasis patients with several studies (Armstrong *et al.* 2011, Lin & Huang 2016, Peluso *et al.* 2016). After psoriasis modeling, oxidant levels were significantly increased along with a less significant increase in antioxidant levels. This was explained by the effect of formation of the 3D system since the better interaction of cells may create a protective environment through different mechanisms. After treatment, oxidant levels decreased significantly, and antioxidant levels increased two-fold suggesting the efficacy of the drug, although the mechanism should be inquired.

In this study, we created the first stress-related psoriasis spheroid model which exhibited correlation in multiple aspects with the literature and searched for the efficacy of a known drug for the first time in a 3D culture system. One of the main novelties of our study is the investigation of a specific β-agonist for determining its efficacy in psoriasis treatment. Although we proved that salbutamol treatment may be a possible therapy for psoriasis, other β-agonists should also be investigated. In our perspective, considering the known effects and the suitability of topical application of β_2 agonists, the efficacy of salbutamol should not be underestimated and must be evaluated further for translation of this knowledge into clinics.

Limitation of the Study

The main limitation of the study is the lack of demonstration of a specific mechanism underlying the changes in gene expression at mRNA and protein levels. Other limitation is the lack of information on if the spherical psoriatic organoids recapitulate the human disease. Lastly, here there are limited numbers of genes studied. Since psoriasis has a complex background affecting more than one pathway, the change in the expression levels of molecules overriding those pathways with disease modeling must be further analyzed.

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