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 ORIGINAL ARTICLE

Novel Strategy for Strengthening Dermatoporotic Skin by Managing Cellular Senescence

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ABSTRACT

Background: Dermatoporosis (DP) is a condition associated with thinning skin layers and resultant fragility. Much of the thinning is related to fibroblast dysfunction, production of destructive inflammatory cytokines, breakdown of the extracellular matrix (ECM), and weakening of the dermo-epidermal junction. A major contributor to this change in the ECM milieu, previously under-considered, is cellular senescence, particularly involving the papillary dermal fibroblasts.

Methods: A series of experiments were undertaken to explore the impact of a combination of known actives on senescent cell status. Human keratinocytes and fibroblasts were cultured, and cytotoxicity tests were performed to determine the ideal concentration to avoid cell toxicity. Microdoses of Centella asiatica (0.005%) and mandelic acid (0.05%) were found to be ideal in avoiding any cytotoxicity. However, the challenge was then to assess the efficacy of these actives in this microdosed form. After exposing the cells to the compounds, RNA was isolated and sequenced. Moreover, a well-described ex vivo model using photodamaged skin was subjected to immunofluorescence to identify senescent cells (via p16INK4a), particularly in the papillary dermis, using the microdose formulation compared with untreated skin. In addition, JAG/NOTCH expression in the epidermal basal cells was evaluated to further understand the cellular senescence signaling mechanism.

Results: Microdosing these two well-known agents had surprisingly significant synergistic effects in vitro, decreasing senescence-associated secretory phenotype (SASP) cytokines and the associated inflammation involved in the process. The ex vivo model revealed a significant ($P<0.05$) decrease in senescent cells in the papillary dermis and a significant increase ($P<0.001$) of JAG/NOTCH expression in the basal cells of the epidermis.

Conclusion: Using microdoses of two known agents, a novel approach produced an unexpected effect of reversal of dermal senescent cells and promoting an anti-inflammatory milieu. A gene expression analysis of the individual and combined actives validated these observations, followed by full formulation testing in an ex vivo model. The approach of limiting cellular senescence in dermal fibroblasts for managing DP is novel and provides an exciting new direction to address dermatoporosis. Clinical studies will follow.

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INTRODUCTION

Dermatoporosis (DP) is characterized by thin, aged, fragile skin.[1] It occurs as part of intrinsic aging but can be hastened by extrinsic factors, such as photodamage, corticosteroid use, and genetic susceptibility. Skin atrophy is characterized by a breakdown of the extracellular matrix (ECM), a lack of support for cutaneous vasculature, and resultant vessel vulnerability.[1-4] DP is especially noticeable on the forearms, dorsal hands, presternal area, scalp, and pretibial areas.[3]

Dermatoporosis shows early changes at approximately 40 years old but develops fully between 70 and 90 years.[2] Dermal thinning is a predictor of a high risk of skin tears among elderly patients,[5] and diminished hyaluronic acid (HA) levels are thought to play a part in dermatoporotic skin.[1,2,6]

Cellular senescence is a less discussed phenomenon associated with dermal atrophy and skin thinning associated

with DP. This is a logical contributor to dermal atrophy and ECM deficiency, as senescent fibroblasts in the dermis no longer produce collagen, elastin, and HA. In addition, this descriptive 'sleeping cell' terminology creates a false narrative as these senescent fibroblasts produce a senescence-associated secretory phenotype (SASP). These products secrete high levels of inflammatory cytokines, immune modulators, and proteases that can create an inflammatory milieu with ECM breakdown and further stimulus for senescent fibroblast formation.[7] In the epidermis, atrophy and thinning are also apparent in DP subjects. Basal cells, responsible for the turnover of epidermal cells, consist of stem cells with high proliferative potential and transit-amplifying cells, which are destined to undergo terminal differentiation after a few rounds of division.[8,9] Although senescence is unusual in the epidermis, it is difficult to differentiate terminal differentiation from senescence. Allied with this, the removal of senescent cells, thought to be undertaken by the innate immune response, may be different in the epidermis due to this unique terminal differentiation sequence, which may be a mechanism for removing senescent cells.

To this end, a strategy of decreasing dermal senescent cells, encouraging defense against senescent epidermal cells, and, more importantly, eliminating these cells is a logical approach to managing DP. Combining an effective emollient base with selected botanical extracts and a hydroxy acid constituent to produce the correct pH formulation, senescent cell investigations were undertaken as a background to DP product formulation. From the aspect of DP, active formulation components were chosen to 'wake up' dormant senescent cells, decrease inflammation, and stimulate cellular turnover. A low pH was identified as important for improving and encouraging the regenerative milieu in aging skin.[10,11] Therefore, one component is an acid (low pH) compound known to be skin-friendly but also to stimulate cellular turnover – mandelic acid. The other component chosen (*Centella asiatica* extract) regulates ECM homeostasis and can affect cellular senescence.[12,13] Initial cytotoxicity studies revealed that extremely low microdoses (0.005% *Centella* and 0.05% Mandelic acid) were needed to ensure cell viability.

MATERIALS AND METHODS

Cell Culture and Compounds

Primary adult normal human dermal fibroblasts and primary adult normal human epidermal keratinocytes were obtained from the American Type Culture Collection (ATCC, Manassas, VA). Each cell type was grown in its respective medium from ATCC. The cells were cultured in a humidified chamber with 5% CO2 at 37°C. *Centella asiatica* extract was from Indena S.p.A, and mandelic acid was from Evonik Operations GmbH.

Cytotoxicity Assessment

Keratinocytes and fibroblasts were plated into a 96-well flat bottom black wall tissue culture plate. For each cell type, 12,000 cells were plated in each well. After 48 hours, the cells were 80% confluent. Then, a cytotoxicity concentration-response study was conducted. The compounds were diluted in media in 2x steps over 12 concentrations. The *Centella* concentrations ranged from 0.000122% to 0.25%. The mandelic acid concentrations ranged from 0.000039% to 0.08%. Cultures were maintained for 72 hours in standard conditions. An Alamar blue assay was conducted, and the fluorescence intensity values were measured in a PerkinElmer Envision 2103 plate reader with 560 nm excitation and 590 nm emission filters.

Cell Treatment and RNA Isolation Preparation

Keratinocytes and fibroblasts were plated into 48-well tissue culture plates (40,000 cells/well). After 96 hours of incubation at standard conditions, the cells were 80% confluent. Then, the medium was changed to generate the following conditions: growth media alone, growth media + 0.005% *Centella*, growth media + 0.05% Mandelic acid, or growth media + 0.005% *Centella* + 0.05% Mandelic acid. After 72 hours , the cells were harvested from 3 wells of each treatment for each cell type with accutase. The collections were pooled into a single vial and snap-frozen at -80 °C.

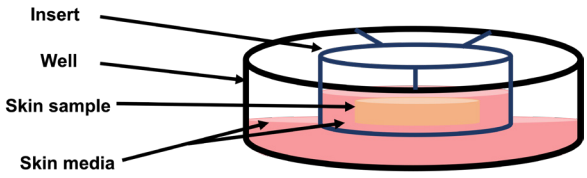
RNA-sequencing (RNA-seq)

The cell pellets were processed at MedGenome, Inc. (Foster City, CA) for RNA isolation, Illumina (San Diego, CA) sequencing library construction, sequencing, and mapping. Each sample was sequenced to ~25M paired reads. All the samples were highly consistent in quality and sequencing metrics. More than 95% of the reads met Q>=30, indicating a high-quality library and sequencing run. The base distribution of the inserts was very close to 25% for each nucleotide, indicating good complexity of inserts. The guanine-cytosine (GC) content was normally distributed around a mean of 50%, as expected for human mRNA. After obtaining the reads, the differentially expressed genes (DEGs) were identified. The DEGs were subjected to the Reactome Pathway Database to assess for pathway enrichment.[14] In addition, a Search Tool for the Retrieval of Interacting Genes/Proteins (STRING) analysis was done to determine potential protein-protein interactions and functional enrichments.[15]

Human Skin Ex Vivo Model

After IRB approval, discarded de-identified human skin samples from patients undergoing facelift procedures were obtained within 2 h of removal from the patients. All skin processing was done under BSL2 laboratory conditions. The skin was washed in phosphate-buffered saline (PBS) and defatted if necessary. Any visible hairs were shaved using a scalpel. The skin was then cut into ~5 mm x 5 mm to ~8 mm x 8 mm square pieces and placed into transwells suspended in 12- well plates and 1.0 ml of Skin Media (DMEM/Ham's F-12 50/50 Mix, Adenine hydrochloride hydrate (50 mM), Calcium Chloride Dihydrate (1.88 mM), T3 Tri-iodothyronine (0.02 nM), Insulin-Transferrin-Selenium-

FIGURE 1. Ex Vivo Model. Discarded skin is cut into ~5 mm x 5 mm to ~8 mm x 8 mm square pieces and placed into transwells suspended in 12-well plates and 1.0 ml of Skin Media is added to each well, and about 200 to 300 ml is added to each transwell to surround the skin sample while maintaining an air-exposed epidermal surface.



Ethanolamine (ITS -X) (1%), Penicillin/Streptomycin (1%), Fetal Bovine Serum (2%), GlutaGRO (1%), and Gentamicin sulfate (0.01 mg/ml)) was added to each well, and about 200 to 300ml was added to each transwell to surround the skin sample while maintaining an air-exposed epidermal surface (Figure 1).

The media was changed daily. The skin in the transwell cultures was maintained under standard conditions in the incubator for 72 hours before initiating treatment. The full emulsion formulation (100-500 ml) was placed on the surface of a sterile petri dish. The skin was retrieved from the transwell culture plate using sharp forceps, picking up the skin at the edge with minimal forceps compression of the skin. The skin was gently swiped over the formulation to cover the epidermis completely and then returned to the transwell culture plate. Untreated skin was used as a baseline control. The treatments were done each day for 7 days.

Immunofluorescence

After treatment, the tissues were fixed in paraformaldehyde, embedded in paraffin, and sectioned. The sections were processed using standard methods and incubated with a primary antibody targeting p16INK4a (Abcam, Fremont, CA), Notch-1 (Cell Signaling, Danvers, MA), or Jagged-1 (Novus, Centennial, CO). Then, fluorescently conjugated secondary antibodies (Jackson Immunoresearch, West Grove, PA) were used to detect target expression. The sections were mounted with ProLong Gold Antifade Mountant with DAPI (ThermoFisher, Waltham, MA). Images were obtained using a Zeiss Axio Observer running Zeiss Zen software. The images were analyzed using ImageJ. Five fields of view were assessed per sample (n =2).

Statistical Analysis

The data from the immunofluorescence imaging were analyzed using a student's t-test. The data are presented as the mean±SD; $P<0.05$ was used to indicate significance.

RESULTS

Cytotoxicity Assessment

The IC_{50} values for *Centella* were 0.05% for the fibroblasts and 0.02% for the keratinocytes. The maximal concentration that did not demonstrate cytotoxicity was selected as the treatment concentration for the subsequent experiments. Thus, 0.005% *Centella* was chosen for both the fibroblasts and keratinocytes (Figure 2A and 2B, respectively). Mandelic acid was significantly less cytotoxic than *Centella* in the in vitro assay. The IC_{50} values for

FIGURE 2. Determining *Centella* and mandelic acid working concentrations. Fibroblasts (A and C) and keratinocytes (B and D) were treated with *Centella* and mandelic acid at varying concentrations to assess cytotoxicity. The x-axis is the reading from the Alamar blue assay, and the y-axis is the concentration. Each treatment was done in triplicate. The arrows indicate the concentrations selected for the subsequent experiments.

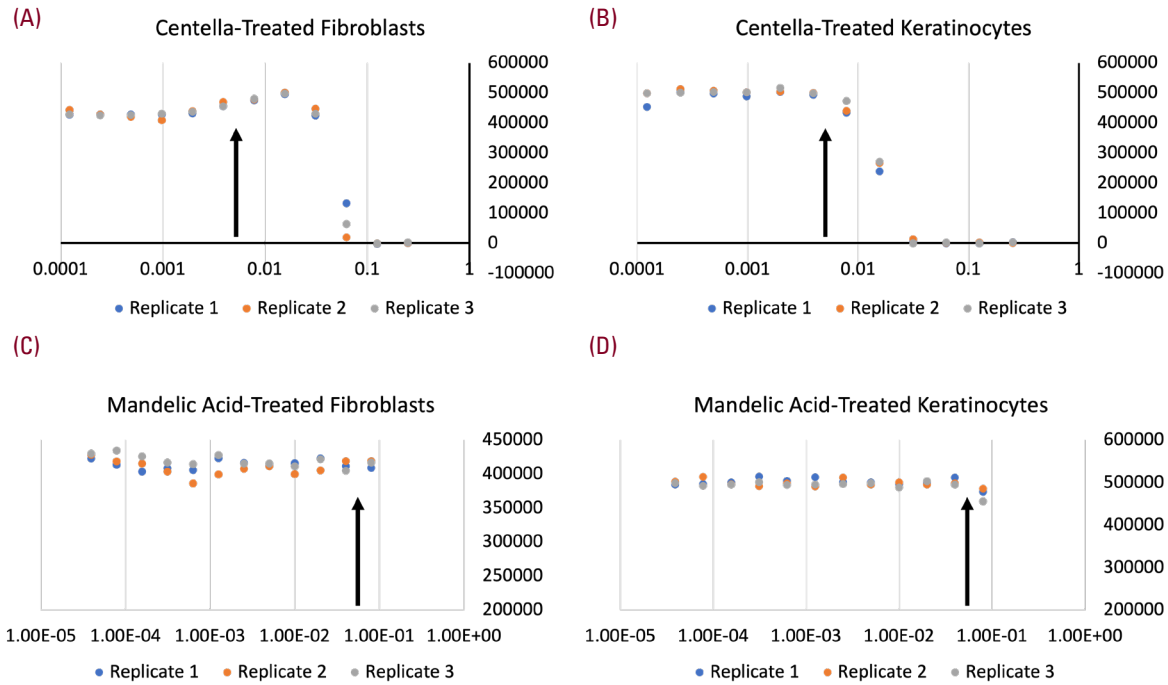


TABLE 1.

Fold Changes in Senescence-Associated Secretory Phenotype-Associated Genes Elaborated by Keratinocytes After Exposure to Individual and Combined Agents

Gene	*Centella*	*P*-value	Mandelic Acid	*P*-value	*Centella*+Mandelic Acid	*P*-value
IL8	-5.50	*P*<0.05	-1.46	ns	-7.17	*P*<0.01
IL1B	-5.05	*P*<0.01	1.73	*P*<0.05	-6.41	*P*<0.01
TNF	-8.62	*P*<0.001	-2.69	*P*<0.05	-7.83	*P*<0.001
HIST1H4H	-4.82	*P*<0.05	-0.54	ns	-4.16	*P*<0.05
HIST1H2BG	-3.98	ns	0.45	ns	-4.68	*P*<0.05
UBE2C	-2.77	ns	0.51	ns	-5.44	*P*<0.05
UBB	-5.06	*P*<0.01	-3.87	*P*<0.001	-5.69	*P*<0.05
UBC	-3.43	*P*<0.05	-1.09	ns	-3.16	*P*<0.05
CEBPB	-4.63	*P*<0.05	-2.45	*P*<0.01	-3.69	*P*<0.05
HIST1H2BD	-4.05	*P*<0.05	0.55	ns	-4.02	*P*<0.05
HIST1H4E	-11.59	*P*<0.05	0.60	ns	-6.62	*P*<0.05
HIST1H3D	-11.21	*P*<0.05	-0.23	ns	-11.00	*P*<0.05

ns = not significant

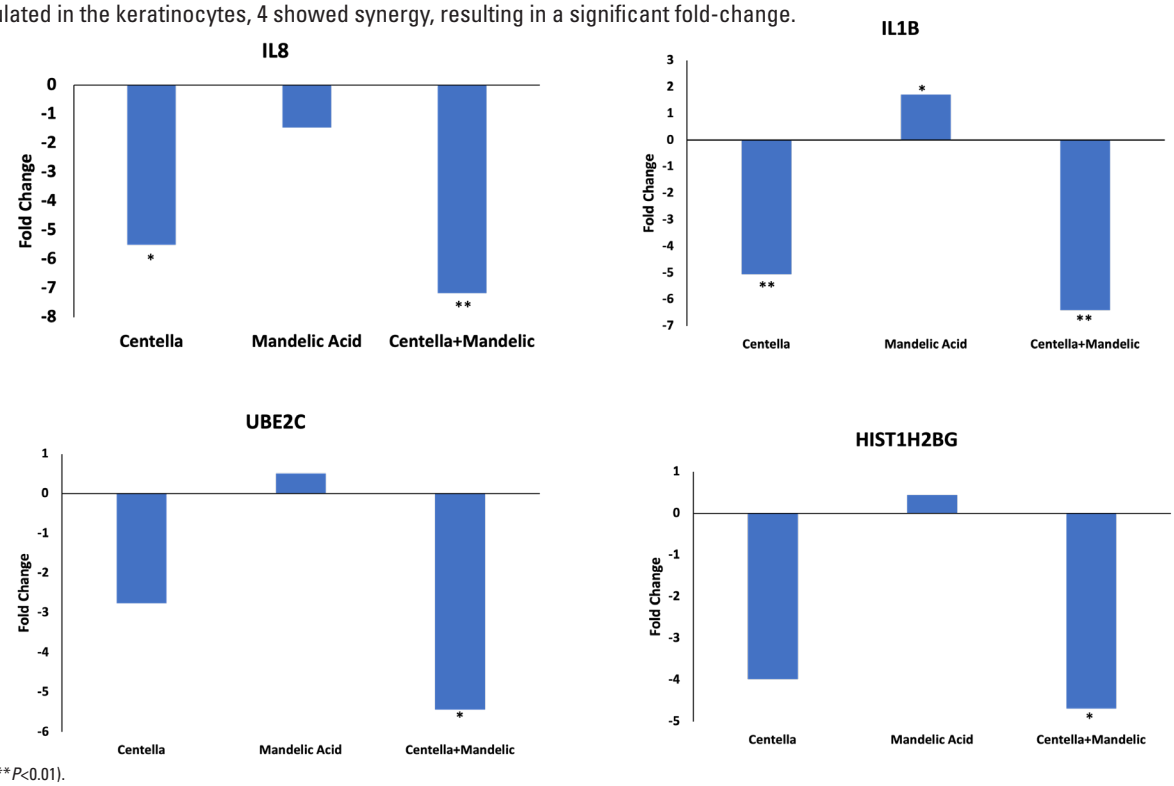
mandelic acid were calculated to be >0.08% for both fibroblasts and keratinocytes. Thus, a mandelic acid concentration of 0.05% was selected for both fibroblasts and keratinocytes (Figure 2C and 2D, respectively).

RNA-seq Results for Keratinocytes

To assess for effects of Mandelic acid and *Centella* individually and together on keratinocytes, DEGs from keratinocytes from these agents individually and together were compared with untreated cells. There was a significant downregulation of genes related to SASP (R-HSA-2559582). The fold changes were compared by examining the single treatment groups and the combined treatment group compared with the untreated cells (Table 1).

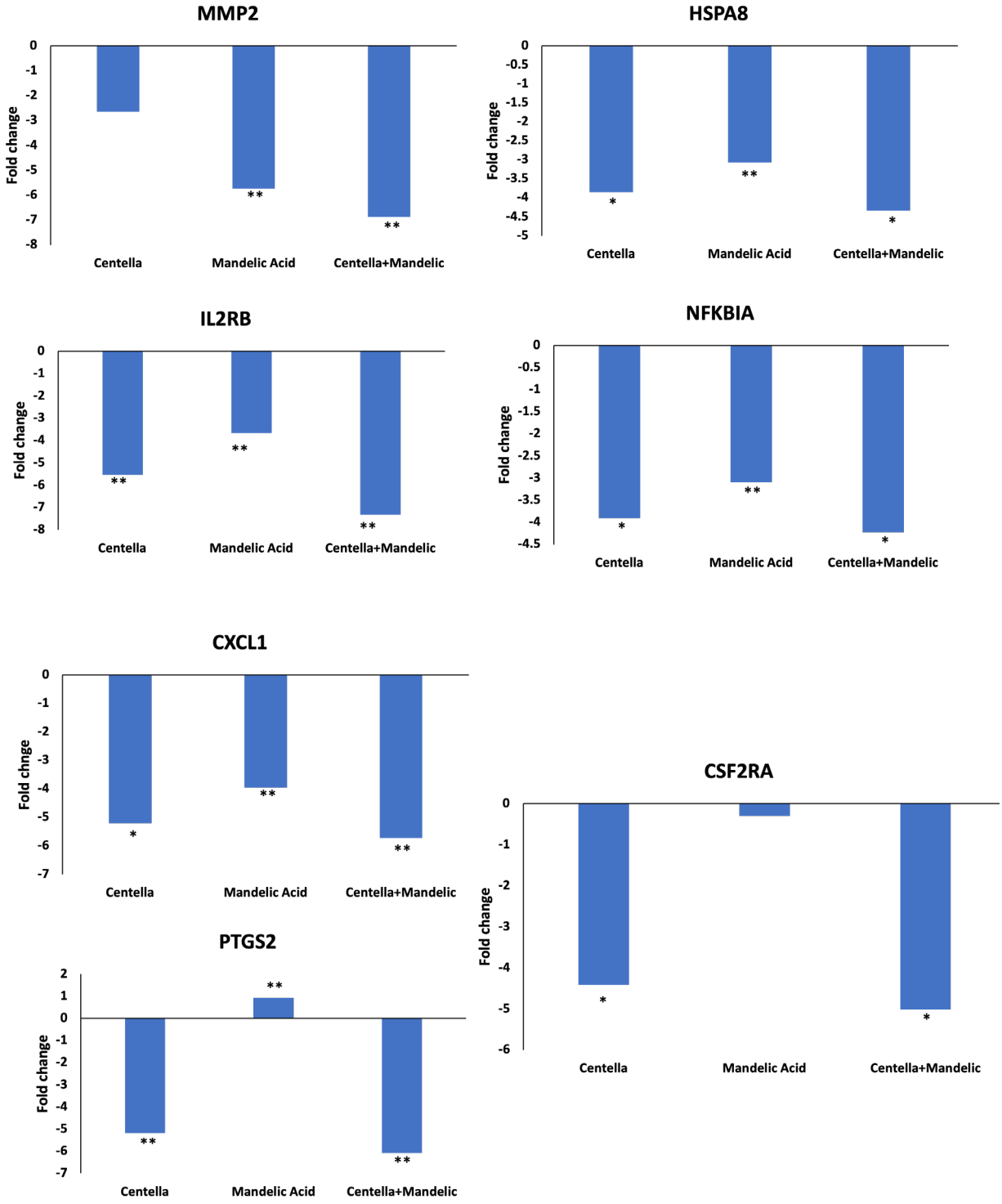
To assess the synergistic effect of *Centella* and mandelic acid, we identified SASP-associated genes that showed an additional downregulation in keratinocytes when the 2 compounds were

FIGURE 3. Senescence-Associated Secretory Phenotype-Associated gene synergy in keratinocytes. Among the 12 SASP genes significantly downregulated in the keratinocytes, 4 showed synergy, resulting in a significant fold-change.



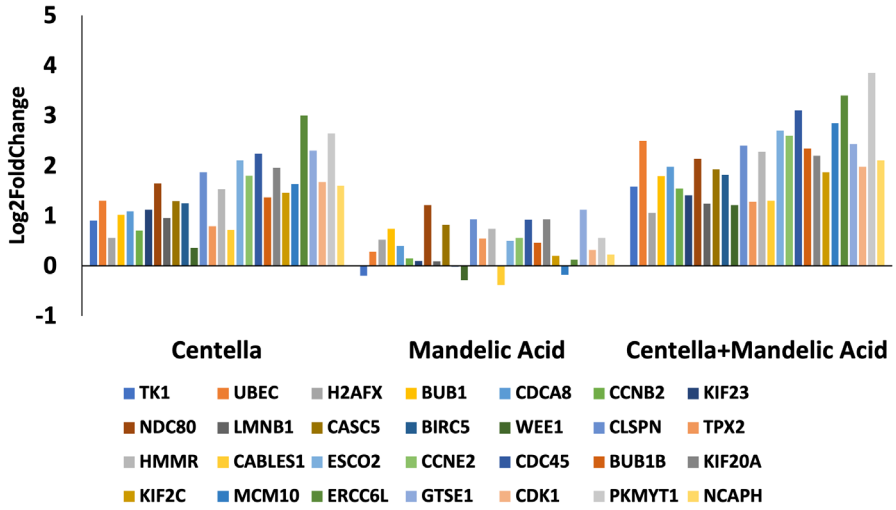
(*P<0.05; **P<0.01).

FIGURE 4. Multiple inflammatory genes are downregulated in keratinocytes with the combination of agents. Among the DEGs identified in the keratinocytes after treatment, 7 genes related to inflammation were significantly downregulated when the cells were treated with both agents. The fold-changes for each treatment are displayed in the graphs for the 7 genes.



(*P<0.05; **P<0.01; compared to non-treated cells).

FIGURE 5. Fibroblasts upregulate cell cycle genes with the combination treatment. Among the DEGs identified in the fibroblasts after treatment, 28 genes related to the cell cycle were significantly upregulated when the cells were treated with both agents.



All the fold-changes for the co-treatment group reach significance ($P<0.05$). None of the single-agent treatment fold changes was significant.

combined. The synergy had to result in a statistically significant downregulation. Four of the genes listed in Table 1 fit these criteria, including interleukin-8 (IL8), IL1B, H2B Clustered Histone 8 (HIST1H2BG), and ubiquitin conjugating enzyme E2 C (UBE2C) (Figure 3).

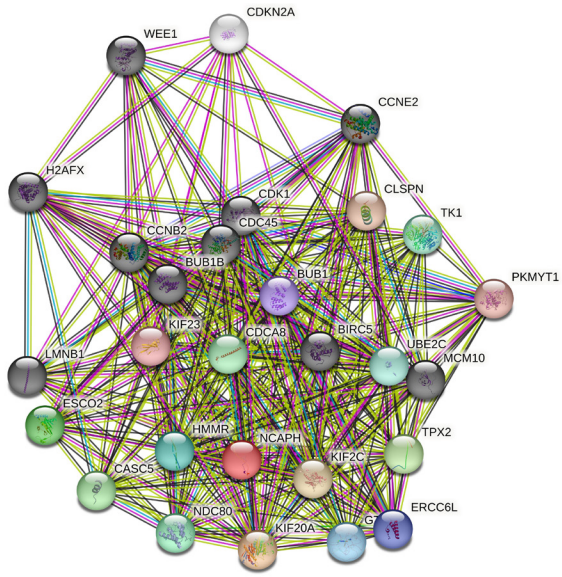
Another pathway enriched in the keratinocyte gene-expression data was inflammation. Among these genes, 7 showed synergy when *Centella* and mandelic acid were combined, including matrix metalloproteinase 2 (MMP2), heat shock protein A8 (HSPA8), Colony Stimulating Factor 2 Receptor Subunit Alpha (CSF2RSA), C-X-C Motif Chemokine Ligand 1 (CXCL1), Interleukin 2 Receptor Subunit Beta (IL2RB), NF-kappa-B inhibitor alpha (NFKBIA), and Prostaglandin endoperoxide synthase (PTGS2) (Figure 4).

RNA-seq Results for Dermal Fibroblasts

Next, the DEGs in the fibroblasts were assessed. The analysis revealed an association with genes related to cell cycle regulation for 28 genes. However, the differential expression of the genes was only significantly upregulated when the treatments were combined (*Centella* + mandelic acid; all $P<0.05$; Figure 5).

A String analysis was conducted to explore the relationship between cell cycle regulation and senescence to find connections between CDKN2A (also known as p16), a senescent cell marker,[16] and the cell cycle DEGs (Figure 6). DKN2A (displayed in white) directly interacted with 10 DEG cycle genes (indicated in black). Since p16 (CDKN2A) blocks cell activity (representing senescence), upregulation of cell cycle genes suggests that p16 activity is decreased. The upregulated cell cycle-associated genes connected to p16 were WEE1, H2A histone family member X (H2AFX), laminin B1 (LMNB1), cyclin B2 (CCNB2), BUB1, cell division cycle 45 (CDC45), cyclin-dependent kinase 1 (CDK1), Survivin (BIRC5), MCM10, and cyclin E2 (CCNE2) (Figure 7).

FIGURE 6. STRING Analysis of upregulated cell cycle genes and p16 (CDKN2A). After treatment with the agents alone or together, the fibroblasts showed an upregulation of genes related to the cell cycle.



A STRING analysis was done to determine if any of these genes were related to the cell senescent marker p16 (CDKN2A; indicated in white). Among the 28 cycle genes, 10 directly interacted with p16 (indicated in black).

Overall Gene Expression Findings

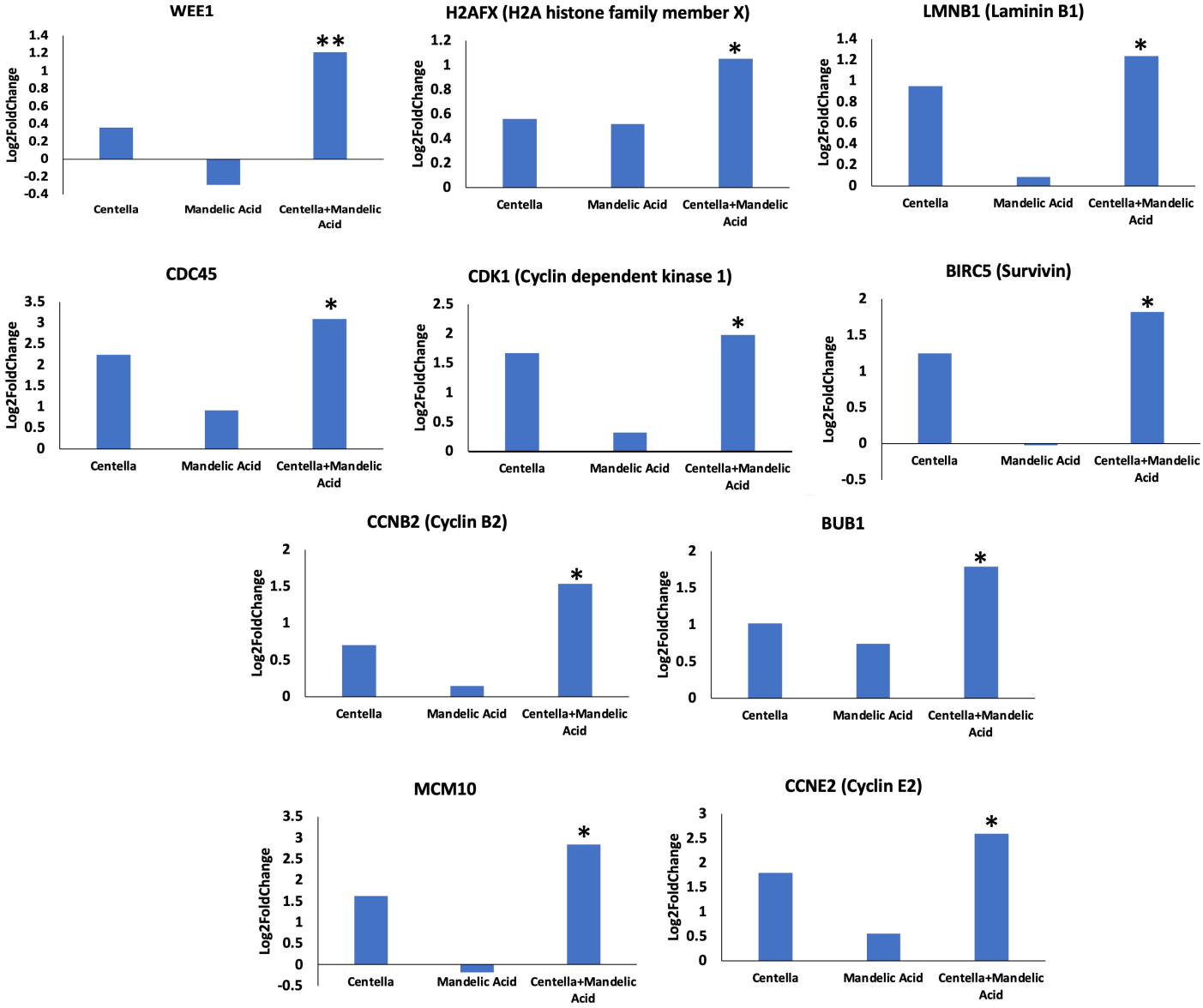
The gene expression data revealed a surprisingly robust synergistic response in the following relevant areas:

- When the agents were combined, genes related to the senescence-associated secretory phenotype (SASP) were significantly decreased in keratinocytes. This combination aids in evading senescent cell activity in keratinocytes.
- For the fibroblasts, genes related to cell cycle activity were upregulated, which suggests increased dermal fibroblast cell turnover when the agents are combined, indicating reduced p16 activity.
- The inactivity of p16 (senescent cell marker, cell cycle blocker) in the fibroblasts, which is connected to an upregulation of cell cycle genes, suggests decreased cellular senescence in fibroblasts with the agents.

Ex Vivo Exploration of Fibroblast Senescence in the Papillary Dermis

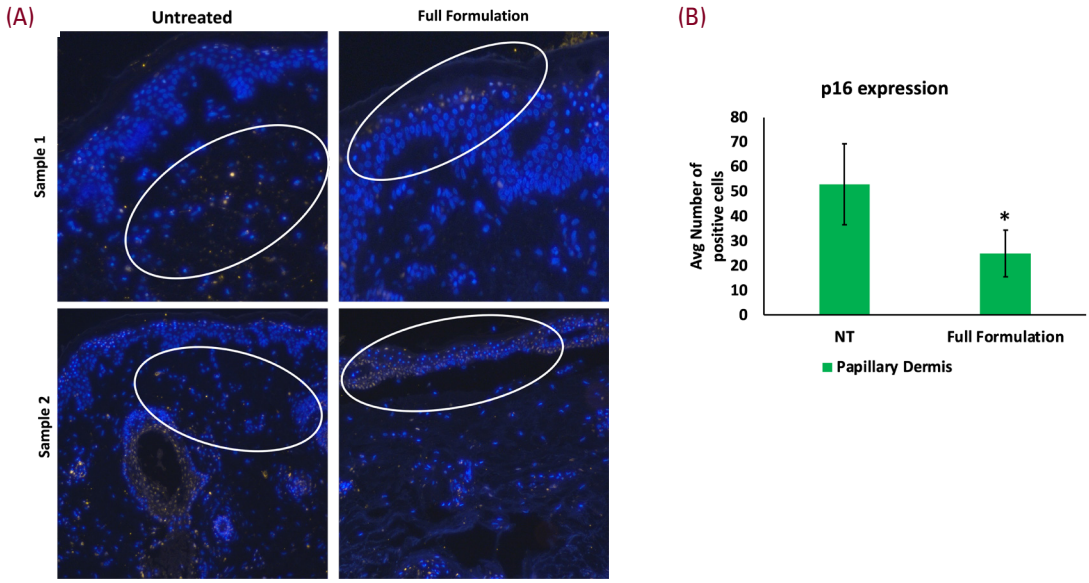
A skin explant ex vivo model was employed to confirm the gene expression findings. The full formulation comprising the microdosed actives of Mandelic acid and encapsulated *Centella asiatica* was used. The delivery system for the *Centella asiatica* extract is comprised of spherical vesicles with a single or multi-

FIGURE 7. Ten cell cycle-associated genes showed a relation to cell senescence in fibroblasts. The log2foldchange of the genes is shown upon treatment with the agents alone or together. The foldchange was only significant when the agents were combined.



(**P< 0.01 and *P< 0.05; compared to non-treated cells).

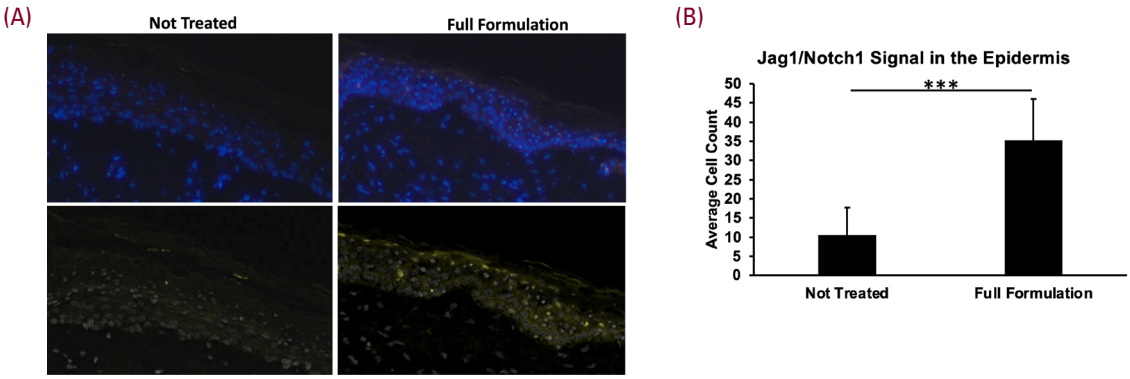
FIGURE 8. p16 Expression assessment with full formulation treatment ex vivo. Skin explants were prepared for ex vivo culture and treated or not treated with the full formulation for 7 days. (A) After fixing and staining the tissue, immunofluorescence analysis was used to detect p16 expression (indicated in yellow) with a DAPI counterstained to detect nuclei (blue). The images are representative of the 2 samples tested (B) The average number of p16 positive cells was determined using ImageJ. Five fields of view were assessed per sample (n = 2) for quantitation.



The data are presented as the mean±SD. *P<0.05.

phospholipid bilayer membrane(s) surrounding an aqueous core representing a skin cell-like composition and structure with a biomimetic carrier. The liposome measures 180-210 nm and entraps lipophilic actives in the membrane and/or hydrophilic actives in the internal core. *Centella asiatica* compound is encapsulated into the inner core of the vesicle and released to the skin, into the stratum corneum, epidermis, and dermis. This full formulation was applied to the skin explants over 7 days (with 3 days of tissue acclamation). p16 expression, as a surrogate for senescence, was detected by immunofluorescence (Figure 8A). The untreated sample showed evidence of senescent cells in the dermis. The treated samples showed a loss of senescent cells (p16 in yellow) in the dermis. Quantification revealed that p16 expression in the papillary dermis was significantly decreased (P<0.05) after treatment (Figure 8B). This finding was repeated using an additional donor (data not shown). Thus, the ex vivo study analysis demonstrated substantiation of p16 senescent activity per the gene expression suggestion. The decreased senescent cells in the dermis also demonstrate probable increased dermal cellular turnover.

FIGURE 9. JAG1/NOTCH1 Expression assessment with full formulation treatment ex vivo. (A) The top panels show the original images (blue for DAPI and Orange for Jag1 (yellow)/Notch1(red). To see the targets more clearly, the DAPI was recolored grey, and the Jag/Notch was recolored yellow, as shown in the bottom panels. (B) The average number of JAG1/NOTCH1 positive cells was determined using ImageJ. Five fields of view were assessed per sample (n = 2) for quantitation.



The data are presented as the mean±SD. ***P<0.001.

Assessment of Jag/Notch Expression in epidermis

Notch signaling is demonstrated to aid in senescent cell removal in the epidermis. There is an age-related decrease of JAG1 expression in the basal layer due to reduced Notch activation.*18* To assess a potential mechanism of senescent cell removal, we employed the same ex vivo model described above. The full formulation was applied to the skin explants over 7 days (with 3 days of tissue acclamation) and NOTCH1 and JAG1 were detected. The top panels of Figure 9 reveal the original images (blue for DAPI and Orange for JAG1(yellow)/ NOTCH1(red). To see the targets more clearly, the DAPI was recolored grey, and the JAG1/NOTCH1 was recolored yellow, as shown in the bottom panels. Quantifying the cells in epidermis positive for JAG1/NOTCH1, revealed a significant increase after full formulation treatment (Figures 9 A and B).

DISCUSSION

The dramatic thinning of the skin and ECM breakdown with resultant fragility characteristic of DP may well result from inactivity and dysfunction of dermal fibroblasts, the cell factory for ECM composition. Cellular senescence and the resultant SASP-induced inflammatory milieu are conducive to ECM breakdown and skin atrophy. A formulation was designed based on the concept of hormesis, a phenomenon whereby exposure to high levels of stressors is inhibitory, but low doses are stimulatory.[17] This is particularly relevant to mandelic acid, where micro-doses well below manufacturer-recommended concentrations were used. Mandelic acid in higher doses promotes cellular exfoliation and concomitant irritation. However, in this micro-dose form and in combination with a micro-dose of *Centella* asiatica, an unexpected anti-inflammatory, anti-senescence effect was demonstrated. This provides an ideal therapeutic pathway for DP management. The hint of this activity was initially provided by cytotoxicity testing, where extremely low doses appeared to be protective of cell survival.

Decreasing senescent fibroblasts in the dermis is a logical strategic approach to reinstating a depleted ECM and thinned dermo-epidermal junction (DEJ). Constant communication takes place between basal epithelial cells, DEJ, and papillary fibroblasts, and reconstituting these important areas by removing reactive senescent cells is an excellent first strategy. It has also been demonstrated that senescent cells are preferentially removed through cell competition via JAG1-NOTCH1 signaling between senescent cells and surrounding normal cells.[18] In the basal cell layer, senescent cells were found to be removed by macrophages in the dermis. To achieve constant removal of senescent cells, it would be important that the cell competition depending on JAG1-NOTCH1 signaling occurs appropriately upon the appearance of senescent cells.[18] Thus, preventing the age-related decrease in JAG1-NOTCH1 expression or replenishing the signaling capacity in these cells works synergistically with the removal of senescent cells. Both scenarios have been demonstrated to have changed positively under the influence of the topical formulation.

CONCLUSION

The design of a formulation directed at dermatoporosis is presented. Combining micro-doses of two active agents showed an unexpected effect of the reversal of dermal senescent cells and promotion of an anti-inflammatory milieu. A gene expression analysis of the individual and combined actives validated these observations, followed by full formulation testing in an ex vivo model. The approach of limiting cellular senescence in dermal fibroblasts for managing DP is novel and provides an exciting new alternative for dealing with this natural phenomenon. Clinical studies will follow.

DISCLOSURES

Dr Chang's contribution to this publication was as a paid consultant and was not part of her Stanford University duties or responsibilities.

John Garruto, Lucien Ionescu, and Drs Widgerow, Zeigler, Shafiq, and Meckfessel are all Galderma employees. These studies were funded by Galderma Laboratories, LP.

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