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“Targeting phosphoproteomic changes in the epidermis to help skin adapt rapidly and efficiently to exposome”

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1. Introduction

The skin is a constantly regenerating tissue, whose dynamics rely on close coordination between structure and function within the epidermis. This dynamism is expressed in particular by the differentiation of keratinocytes, which migrate from the basal layer to the horny layer, undergoing morphological and functional transformations. This process involves a continuous reorganization of the cellular architecture, in particular of the adhesion junctions. These junctions are dynamic structures that ensure keratinocyte motility and regulate epidermal differentiation and repair processes in real time. Thus, epidermal dynamism relies on a fine and regulated orchestration of adhesive, cytoskeletal, and differentiative interactions.

The epidermis, which is constantly renewing itself, is also constantly attacked by changing environmental stimuli (UV, pollution and temperature variations). To maintain its integrity, the epidermis constantly mobilizes rapid, coordinated and adaptive cellular responses. This dynamic communication relies on the cellular vitality of keratinocytes, which have the ability to perceive, integrate and respond to external signals. One of the key mechanisms of this adaptability is protein phosphorylation, a rapid, reversible and highly regulated post-translational modification. Phosphorylation acts as a switch that activates or inhibits protein activity, allowing keratinocytes to react in real time to changes in their environment. The phosphorylation state therefore reflects the functional vitality of keratinocytes and their ability to maintain tissue homeostasis in the face of aggression. Phosphorylation controls fundamental functions such as proliferation, differentiation, stress response or cell survival and tissue repair.

Junctional and cytoskeletal proteins are highly regulated by phosphorylation, which constitutes a central lever in the orchestration of adaptive responses of the epidermis, allowing the maintenance of a dynamic balance between cellular cohesion, barrier function, and tissue plasticity. However, during aging, this regulation becomes impaired, leading to a loss of reactivity, dynamism, and cellular vitality of keratinocytes. This progressively compromises their ability to maintain cellular cohesion (loss of flexibility of adhesion junctions), to respond to external aggressions (loss of adaptation), and to ensure effective regeneration of the epidermis. Finally, the integrity and resilience of the aged epidermis is compromised in the face of environmental stresses.

When evaluating the efficacy of a cosmetic active ingredient on *in vitro* models of human keratinocytes, it is particularly relevant to measure its impact at the protein level to directly access the functional level. In addition, many proteins become active after post-translational

modifications, of which phosphorylation is one of the most decisive. Therefore, combined proteome and phosphoproteome analysis allows us to be more predictive of the true biological effect of an active ingredient, by demonstrating both the synthesis and functional activation of the targeted proteins.

Using this innovative *in vitro* approach to assess the efficacy of cosmetic products, we selected a new active ingredient. To differentiate total proteins expressed and those engaged in activation pathways by phosphorylation, we had a precise and functional reading of biological effects induced by the active ingredient. After bioinformatics processing with an enrichment analysis of functional terms from gene ontology (GO), we identified the biological processes activated by the extract. Using this multi-omics approach, we showed that the active ingredient reinforced cohesion and skin barrier function. These results were applied to a tissue engineering model subjected to dry cold climate stress. We highlighted that the active ingredient played a role in the dynamism of the epidermis to adapt and respond in real time to environmental stresses and maintain tissue renewal by increasing the expression of filaggrin and strengthening the integrity of skin barrier function.

2. Materials and Methods

2.1. Monolayer cultures of normal human keratinocytes

Normal human keratinocytes (NHK) were extracted from abdomen biopsies of Caucasian female donors. Human skin tissue biopsies were obtained with the informed consent of patients undergoing surgical discard, in accordance with the ethical guidelines of the Tissue Bank Institute (Edouard Herriot Hospital, Lyon, France). NHK were cultured at 37°C in 5% CO₂ in keratinocyte serum-free medium (KSFM) (Gibco™, Thermo Fisher Scientific, Waltham, Massachusetts, USA) supplemented with human recombinant epidermal growth factor (rEGF) at 5 ng/ml and bovine pituitary extract (BPE) at 50µg/ml (Thermo Fisher Scientific). Cells were treated with the active ingredient added in the culture medium at 0.25% for 48h for proteome and phosphoproteome analysis.

2.2. Protein extraction and quantification

After being washed, cells were scraped with cold TBS (Tris Buffered Saline) to extract proteins. The protein material was first cold centrifuged at 2000 g for 10 minutes, then at 500g for another 10 minutes. After removing residues, proteins were recovered, frozen in liquid nitrogen, and stored at -80°C until use. Frozen protein material was lysed in a hot buffer (95°C) (6M guanidine hydrochloride, 40mM chloroacetamide, 10mM TCEP, 100mM TrisHCl pH8.5) then successively boiled and sonicated by micro cavitation (Bioruptor Pico, Diagenode). Protein lysates concentrations were finally determined by the Pierce™ BCA (BiCinchoninic acid Assay) assay.

2.3. Proteome and phosphoproteome analysis

- Samples treatment:

For each sample, 1.1 mg of already denatured proteins were sequentially digested by LysC and trypsin enzymes. Peptides were then purified by SPE chromatography (SepPak tC18), dried and solubilized. Peptide concentration of each sample was determined using Pierce™ Quantitative Colorimetric Peptide Assay. At this step 10 µg of total peptide were kept for label free whole proteome study and 1 mg of total peptide was kept for phosphopeptides enrichment described below.

- Phosphopeptides enrichment:

Phosphopeptides were enriched using metal oxide affinity chromatography (MOAC). An equal volume of 80% ACN (Acetonitrile) - 12% TFA solution was added to each sample and one fifth volume of TiO₂ beads. Samples were incubated for 15min at 30 °C with orbital shaking of 2000 rpm. TiO₂ beads were collected using centrifugation and supernatants were incubated twice more with fresh TiO₂ beads. TiO₂ beads were loaded onto C8 StageTips. The beads were washed and then phosphopeptides were eluted from beads. All fractions were mixed and dried in a SpeedVac. Dried phosphopeptides were dissolved in 50% Isopropanol – 0.5% TFA and then loaded onto SDB-RPS StageTips to be purified according to manufacturer instructions (Phoenix 96X Preomics GmbH). Peptide concentration of each sample was determined using Pierce™ Quantitative Colorimetric Peptide Assay.

- LC-MS/MS (Liquid Chromatography coupled to tandem Mass Spectrometry)

- Whole proteome study

300ng of peptides were injected in triplicate for each sample. Chromatography was performed using Ultimate 3000 (Dionex, Thermo Scientific, Rockford, IL, USA) equipment using PepMap™ 100 C18 (75µm x 50cm, 2µm material) column. Data were acquired using a Q-Exactive (Thermo Scientific) mass spectrometer. MS scan was performed with a resolution of 70000 and an accumulation time of 60ms. MS/MS scan was performed with a resolution of 17500 on the 10 most intense ions of each cycle with an accumulation time of 60ms. 6600 cycles were performed, thus an average of 23 cycles per chromatographic peak.

- Phosphoproteome study

400ng of phosphopeptides enriched extracts were injected in triplicate for each sample. Chromatography was performed using chromatography apparatus. Data were acquired using the same mass spectrometer. MS scan was performed with a resolution of 70000 and an accumulation time of 60ms. MS/MS scan was performed with a resolution of 35000 on the 10 most intense ions of each cycle with an accumulation time of 108 ms. 6287 cycles were performed, thus an average of 20 cycles per chromatographic peak.

- Data analysis :

- Whole proteome study

Proteins were identified using SEQUEST-HT algorithm following MS Amanda algorithm for spectra unidentified by sequest against a database gathering Human reference proteome and cRAP contaminant excluding human proteins databases mined from UNIPROT. False Discovery Rate (FDR) determination was made using Percolator algorithm. The relative quantification was performed by area-based label free quantification using Minora algorithm and Precursors Ions quantifier node for proteome discoverer. We compared protein expression levels, setting a significance threshold ($p\text{-value} \leq 0.05$) and a fold change ($FC > 2$). We therefore analyzed the differential relative expressions of the identified and quantified proteins in keratinocytes treated with the extracts. Untreated keratinocytes were used as a control to establish the reference basal expressions of the proteins.

- Phosphoproteome study

Identification of unlabeled proteins was performed as described for proteome analysis. Validation and localization of unlabeled phosphosites on proteins were performed using the

ptmRS algorithm. Quantification of unlabeled phosphosites was performed in the same manner as protein quantification and as described above.

2.4. Culture of reconstructed human epidermis

Keratinocytes from juvenile foreskin (< 2 years old) were seeded (2×10^5 cells/cm²) onto Millicell® cell culture inserts, 12 mm, polycarbonate, 0.4 µm (Merck Millipore, Burlington, Massachusetts, USA) and cultured in CnT-Prime (CELLnTEC, Bern, Switzerland) at 37°C in 5% CO₂ for 3 days. Then, the system was lifted at the air-liquid interface and the culture was grown in CnT Prime Airlift medium (CELLnTEC) for 18 additional days with a change of the culture medium every 2 days. Human reconstructed epidermis were then treated in preventive with the active ingredient at 0.5% for 48h before subjected to 15-minutes dry cold stress for 3 consecutive days (10°C, 40% Relative Humidity, RH) and treated concurrently for 48h with the active ingredient at 0.5%.

2.5. Immunofluorescence staining

Reconstructed epidermis were fixed using Antigenfix solution 4% (Microm Microtech, Francheville, France) and included in paraffin and cut by the Novotec company. The sections were then deparaffinized in ethanol baths, saturated before processing immunostaining. Primary antibody was directed against filaggrin (rabbit polyclonal antibody, reference HPA030188, Sigma-Aldrich, Saint-Quentin Fallavier, France). Goat anti-rabbit Alexa Fluor 555 (Thermo Fisher Scientific, reference A-21429) was used as conjugate. The mounting medium (Prolong Gold antifade reagent, Thermo Fisher Scientific) contained DAPI for nucleus staining. Fluorescent immunostaining was observed using an Axio Imager M2 fluorescence microscope (Zeiss, Oberkochen, Germany) and quantified (area of ROI) using Zen image analysis software (Zeiss). Filaggrin expression was analyzed by immunofluorescence in % relative to the total surface area in µm². Unstressed epidermis were used as control. The representative data were shown as mean ± SD, in % of filaggrin expression relative to control, from three independent experiments with an analysis of five images per experiment (n=5). The statistical analysis was assessed vs. the dry cold stress condition using nonparametric Kruskal-Wallis test, **** p<0.0001; *** p<0.001.

2.6. Lucifer yellow (LY) permeability assay

Reconstructed epidermis samples were loaded with 150µl of LY dye solution 1mM (Thermo Fisher Scientific) and incubated for 1 hour at 37°C before being rinsed in phosphate buffered saline and processed for fluorescence analysis. Samples were embedded in Tissue Tek OCT compound (Microm Microtech) and frozen sections were cut, placed onto Superfrost Gold plus (Dutsher, Bernolsheim, France) slides and fixed. The mounting medium (Prolong Gold antifade reagent, Thermo Fisher Scientific) contained DAPI for nucleus staining. Fluorescent staining was observed using an Axio Imager M2 fluorescence microscope (Zeiss) and quantified (area of ROI) using Zen image analysis software (Zeiss). Unstressed epidermis were used as control. The representative data were shown as mean ± SD, in % of LY fluorescence expression relative to control from three independent experiments with an analysis of three images per experiment (n=3). The statistical analysis was performed vs. the dry cold stress condition using One-Way ANOVA followed by Tukey's multiple comparisons test, **** p<0.0001, * p<0.05.

2.7. Trans-epithelial electrical resistance (TEER) assay

Integrity of the epithelium barrier was evaluated by measuring the TEER of the reconstructed epidermis (in Ohms) in real time using a calibrated system composed of 2 electrodes

(Millicell ERS-, Millipore/Merck, Molsheim, France). The representative data were shown as mean \pm SD, in % relative to dry cold stress, from three independent experiments in triplicate (n=3). The statistical analysis was performed vs. the dry cold stress condition: Nonparametric Kruskal-Wallis test. ** $p < 0.01$.

3. Results

1.1. Phosphoproteomics: a powerful tool for uncovering protein deregulations not highlighted in classical proteome analysis.

We tested the efficacy of six extracts on keratinocyte monolayers using proteomic and phosphoproteomic analyses. For the whole proteome study, we edited, *i.e.* identified and quantified, more than 3,000 proteins per sample. For the phosphoproteome study, we identified and quantified, more than 9000 phosphosites per sample. An average of $3,049 \pm 198$ proteins were identified in cells. After treatment with actives, the proteomic dataset revealed that $2.42 \pm 0.65\%$ of total proteins exhibited modified expression ($FC > 2$; $p\text{-value} \leq 0.05$). In addition, we showed that $3,010 \pm 354$ phosphopeptides were modified ($FC > 2$; $p\text{-value} \leq 0.05$), affecting $33 \pm 2.5\%$ of total proteins, thus highlighting hidden processes, identified thanks to the phosphoproteome dataset (Figure 1).

	Number of identified proteinnes	Percentage (%) of deregulated proteins (FC \geq 2; $p\leq$ 0.05)	Number of deregulated phospho-peptides	Percentage (%) of impacted proteins with phosphorylation deregulation
6 Active ingredients	3049 ± 198	$2.42 \pm 0.65\%$	3010 ± 354	33 ± 2.5

Figure 1. Proteome and phosphoproteome analysis in active ingredients-treated keratinocytes. Proteome and phosphoproteome were analyzed in a label-free manner by HPLnano-C coupled with tandem mass spectroscopy. The representative data were shown as mean \pm SD of six active ingredients.

1.1.1. Proteome analysis of the active ingredient

To interpret the dataset and identify statistically relevant biological pathways of interest, we used Gene Ontology (GO) enrichment analysis. We decided to study the proteome of keratinocytes treated with the active ingredient whose expression of the greatest number of proteins was modulated.

GO bioinformatics analysis revealed that keratinocytes treated with the active ingredient were enriched in proteins involved in cohesion and skin barrier function compared to control with in particular a very significant increase in the expression of filaggrin ($FC=5.954$), keratinocyte differentiation-associated protein (KRTDAP) ($FC=3.233$) and also dermokine (DMKN) ($FC=2.142$) (Figure 2).

Poteins	Fold change vs. control	Functions
Filaggrin (FLG)	5.954	Differentiation Horny envelopes Barrier function
Keratinocyte differentiation-associated protein (KRTDAP)	3.233	
Involucrin (IVL)	1.896	
Suprabasin (SBSN)	1.986	
Cornifin-A (SPRR1A)	1.578	
ATP-binding cassette sub-family A member 12 (ABCA12)	1000	
Protein-glutamine gamma-glutamyltransferase K (TGM1)	1.919	
Dermokine (DMKN)	2.142	
Protein S100-A9 (S100A9)	1.665	Differentiation Barrier function Inflammation

Figure 2. Proteome or the "biological significance" of the extract active efficacy. Enriched content of proteins of interest in monolayer cultures of human normal keratinocytes treated with the active ingredient at 0.25% for 48h. Untreated keratinocytes were used as control.

1.1.2. Phosphoproteome analysis of the active ingredient

We studied the phosphoproteome of keratinocytes treated with the active ingredient by GO bioinformatics analysis. Using GO analysis of the phosphoproteins regulated by the active ingredient, we highlighted four statistically relevant biological pathways of interest, enriched in active ingredient-treated-keratinocytes: the PI3K/Akt, mTORC1 and mTORC2, PAK and MAPK pathways (Figure 3). Therefore, keratinocytes appeared to exhibit increased mobility and cell adhesion dynamics (anchorage junctions and keratin filament networks) and improved barrier function (cornified envelope) against external stresses.

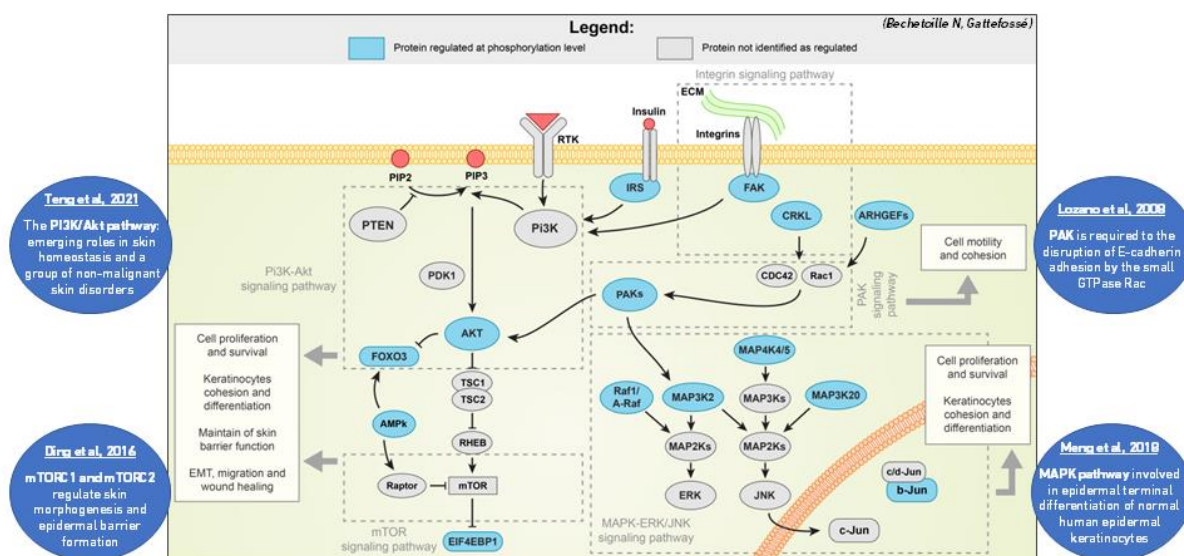


Figure 3. Modulation of canonical pathways in monolayer cultures of keratinocytes treated with the active ingredient at 0.25% for 48h.

Using a more in-depth bioinformatic study on the promising active ingredient, we identified the key signaling event, phosphorylation of PKP1 (S191) probably by phosphorylated PKCα (S226) through a calcium sensing involving down-phosphorylated STIM-1 (S257) (Figure 4).

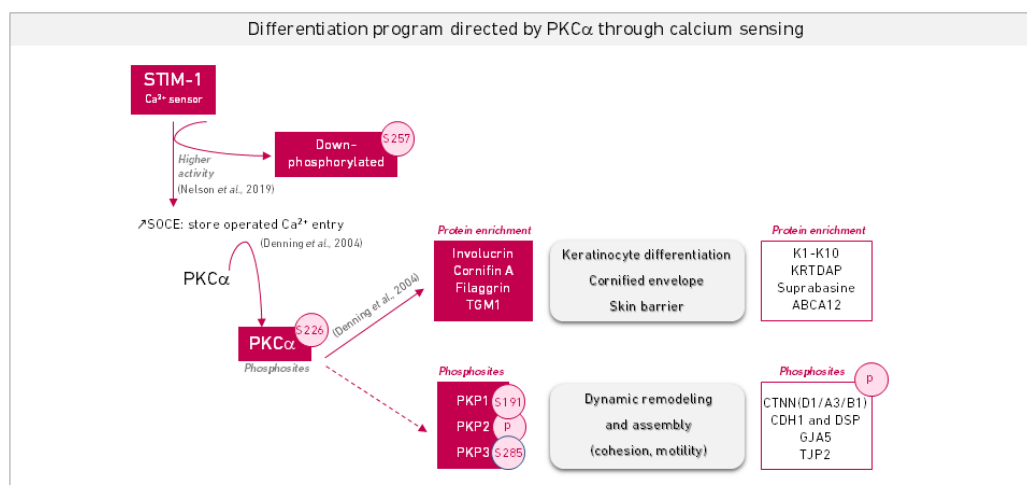


Figure 4. The signaling pathway impacted in the active ingredient-treated keratinocytes.

1.2. The active ingredient boosted adaptation abilities of the skin tissue against the climate stress

Using 3D models of reconstructed epidermis subjected to cold and dry climate stress (10°C, 40% RH), we implemented the results obtained by phosphoproteome analysis and thus confirmed that the active ingredient helped the skin adapt to climate change and maintain tissue renewal by increasing filaggrin expression and strengthening the integrity of the skin barrier function.

1.2.1. The active ingredient increased the filaggrin expression

Exposure of dry cold stress induced a decrease in filaggrin expression in the upper layers of the epidermis (Figure 5.a). And interestingly, treatment with the active ingredient strongly increased filaggrin expression in stressed epidermis. Filaggrin expression was significantly decreased by $-51\% \pm 20\%$ ($p < 0.001$) in stressed epidermis (Figure 5.b), while in the stressed epidermis treated with the active ingredient (0.5%), it increased by $160\% \pm 44\%$ ($p < 0.0001$). Therefore, the active ingredient could help the epidermis adapt to climatic stresses in real time by strengthening the cohesive structure of the tissue.

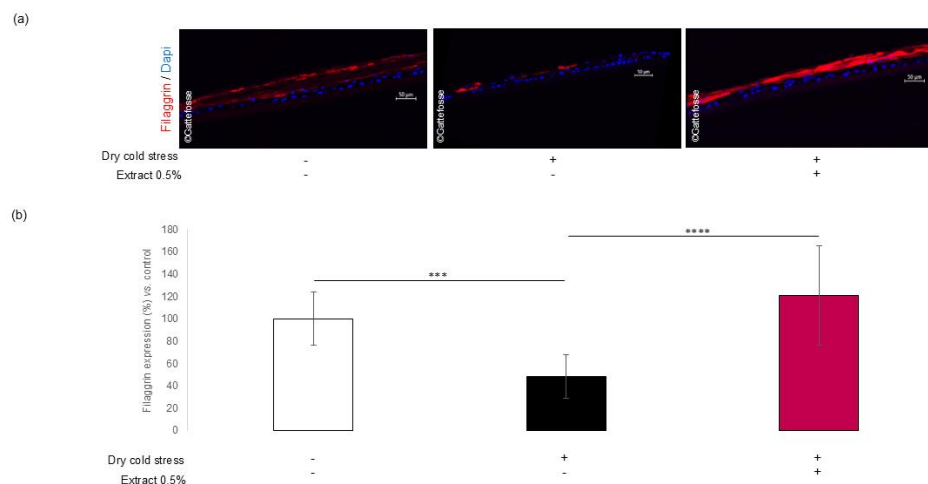


Figure 5. The active ingredient increased filaggrin expression. (a) Immunofluorescence imaging of filaggrin expression (red) in human reconstructed epidermis subjected to climatic stress and treated with the active ingredient at 0.5%. DAPI (blue) was used for nuclear staining. Scale bar = 50µm. (b) Immunofluorescence image analysis of filaggrin expression.

1.2.2. The active ingredient restored the barrier function

Filaggrin is a crucial protein for the correct formation and function of the skin barrier. We therefore examined whether the active ingredient was also able to strengthen the skin barrier function. Exposure of dry cold stress impaired the barrier function of epidermis, as shown by diffuse LY staining in the tissue (Figure 6.a). Interestingly, the LY dye in the stressed epidermis treated with the active ingredient remained localized in the *stratum corneum* (SC) as in the control condition. The LY expression was significantly decreased by $-50\% \pm 35\%$ ($p < 0.05$) in stressed epidermis (Figure 6.b), while in the active ingredient treatment (0.5%), it increased by $199\% \pm 48\%$ ($p < 0.0001$).

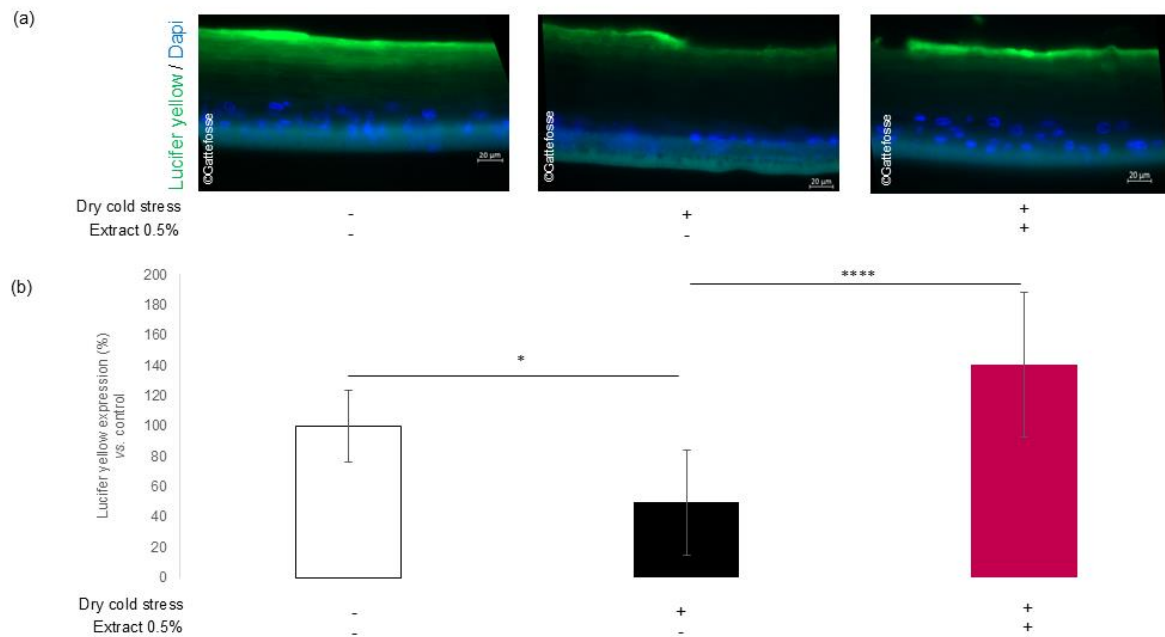


Figure 6. The active ingredient reinforced the skin barrier function. (a) Diffusion of the Lucifer Yellow (LY) fluorescent dye (green) was used to visualize the permeability of reconstructed epidermis subjected to climatic stress and treated with the active ingredient at 0.5%. Scale bar = 20 μm. (b) Quantification of LY fluorescence expression.

To quantitatively assess the integrity of the skin barrier, we used TEER as a permeability assay in the 3D model of reconstructed epidermis subjected to dry cold stress. The active ingredient induced a significant increase in TEER by $+109\% \pm 48\%$ ($p < 0.01$) in stressed epidermis compared to the dry cold stress condition (Figure 7).

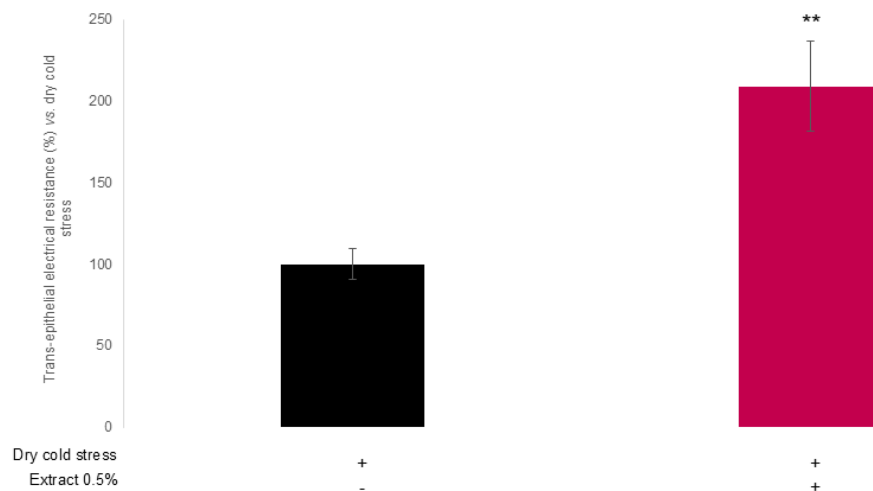


Figure 7. The active ingredient improved the trans-epithelial electrical resistance (TEER). Measurement of TEER (%) in reconstructed epidermis subjected to climatic stress and treated with the active ingredient at 0.5%.

4. Discussion

In cosmetic and dermatological research, the evaluation of active ingredients is mainly based on proteome analysis, *i.e.* the detection and quantification of proteins expressed by skin

cells. However, this approach has a major limitation: it only reflects the presence of proteins, without providing information on their functional activation state. However, most proteins exert their biological role once activated by post-translational modifications, and in particular by phosphorylation. Furthermore, the epidermis is a living organ in constant renewal, whose functions are dynamically regulated. The tissue is also constantly exposed to external aggressions (UV, pollution, temperature variations, etc.) requiring rapid and adaptive biological responses. These responses are based on intracellular mechanisms that are finely regulated over time, often invisible via simple proteomic analysis. The failure to take these dynamic regulations into account in current evaluation methods leads to a partial and potentially inaccurate representation of the real biological effects of cosmetic products. Consequently, there is an unmet need for devices, methods or approaches that can more relevantly and functionally measure the impact of products on the dynamic mechanisms of the skin

To assess the efficacy of active ingredients, we proposed an innovative approach based on a combined analysis of the proteome and the phosphoproteome. This strategy allowed us to consider both the presence of proteins and their activated functional state in the biological model studied. We showed that the active ingredient increased the phosphorylation state of proteins involved in all keratinocyte adhesion junctions, namely anchoring adherens such as plakophilins (PKP1, PKP2 and PKP3) and desmoplakin (DSP), which could affect their recruitment to the junctions and their binding to keratin intermediate filaments [1]. The active ingredient also induced phosphorylation of adherens junction proteins such as E-cadherin (CDH1), β -catenin (CTNNB1), CTNND1 (p120-catenin) and CTNNA2 (α -catenin) which could modify their interactions with the actin cytoskeleton and influence intracellular signaling, differentiation and migration of keratinocytes [2]. Finally, the active ingredient induced the phosphorylation of tight (TJP2) and GAP junction proteins (GJA5), which could affect permeability and communication between keratinocytes, widely described in literature studies [3,4]. By modulating the phosphorylated state of adhesion junction proteins, the active ingredient promoted the dynamic cohesion of cell/cell interactions. Moreover, the active ingredient stimulated the functional vitality of keratinocytes, their ability to perceive and adapt to external signals through the fine modulation of phosphorylation, to ultimately maintain tissue homeostasis.

The stimulation of the functional dynamism of keratinocytes by the active ingredient was confirmed in a more complex 3D model of reconstructed epidermis subjected to dry cold stress to demonstrate the ability of the skin to adapt and respond in real time to environmental stresses in order to maintain renewal and integrity of the tissue. We showed that the active ingredient helped the skin adapt to dry cold stress by strengthening the cohesive structure of the tissue through increased filaggrin expression. To confirm the dynamic cohesion of cell/cell interactions within the epidermis, analyses of expressions of junction proteins such as corneodesmosin, ZO-1 and occludin are in progress in our laboratory. In the SC, filaggrin is proteolyzed into highly hygroscopic free polar amino acids, trans-urocanic acid (UCA) and pyrrolidone-5-carboxylic acid (PCA), which are part of the skin's "natural moisturizing factors (NMF)" and ensure hydration of the SC on the surface [5]. Thus, in our study, the increase in filaggrin expression in the presence of the active ingredient could be accompanied by an increase in NMF. In patients with atopic dermatitis, skin loses its ability to adapt to climate changes and regulate filaggrin expression. As a result, the epidermal barrier is more altered, and the disease worsens [6]. Besides, it has recently been shown that the rise in allergic disorders like AD has been linked to environmental changes [7].

5. Conclusion

Our approach combining bioinformatics and tissue engineering allowed us to decipher dynamic changes, essential for the epidermis to cope and adapt in real-time to its environment.

This original work represents a leap forward in our ability to discover novel actives that will help the skin adapt in real time to climate changes.

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