Identification of skin benefits of a *Lepidium meyenii* root extract on reconstituted epidermis, using whole transcriptome profiling by DNA microarrays and a new *Skin Knowledge Database*

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SUMMARY

The objective of the study was to address *in vitro* the effects of a *Lepidium meyenii* root extract on epidermal benefits. The selected approach was based on human reconstituted epidermis and on whole transcriptome profiling coupled to a skin-specific downstream proprietary data analysis platform.

INTRODUCTION

Gene expression profiling is more and more used in cosmetic research as a tool to support claims, identify novel targets and collect mechanistic data about products in the development phase. It also proved to be very useful to find new activities for existing products, leading to an extension of the product life cycle.

Coupled to cell culture or skin tissue engineering, qRT-PCR in 384-well plate format (TaqMan qPCR arrays) as well as full genome DNA microarrays are the most relevant and friendly technologies to monitor gene expression changes in a medium- or a high-throughput format respectively.

qRT-PCR arrays present the advantage to focus on a relative small but relevant number of genes, identified as key members of skin-specific pathways and process such as differentiation, desquamation, water retention, pigmentation, defence, etc. A deep knowledge in skin biology and/or an in-depth literature review is needed to give sense to the data and identify relevant results in connection with a possible beneficial effect on the skin. Gene expression data are robust and do not need further confirmation by individual qRT-PCR. However, one must keep in mind that the analysis focuses on maximum 384 genes, and do not cover all genes that are expressed in the skin and that might be modulated by the test compound.
DNA microarrays such as the Affymetrix GeneChip Human Gene 2.0 ST present the capability to screen most of the genes that are expressed in a given situation, *i.e.* the whole transcriptome. Data acquisition is based on cDNA hybridization on small gene-specific oligonucleotides. This is a very powerful technique for which there is no *a priori*. It is also important to notice that the results need to be confirmed by qRT-PCR.

Nevertheless, given the massive amount of information, data processing and interpretation might be tricky and time-consuming. The analysis requires computational biology to identify the most relevant data, based on group and individual annotation/analysis. The commercially available databases and web interfaces present the disadvantage to be very generalist and not relevant to skin-related pathways/biology.
This is why a new database concept has been developed in combination with a convenient and friendly interface. The "*Skin Knowledge Database*" (StratiCELL, Belgium), incorporates among 3000 key genes listed into themes and groups specifically related to skin biology and used for data annotation and visualization. A whole expertise on skin biology and product testing is thus now included in a custom, easy to use, web interface connected to a relational database.

This new tool is therefore a key to identify and characterize genes/pathways that are either up-regulated or down-regulated in normal or specific conditions (aged skin, inflamed skin, UV challenged, etc.) and that could be modulated or reversed by the test product, in regards to specific cosmetic benefits/claims.

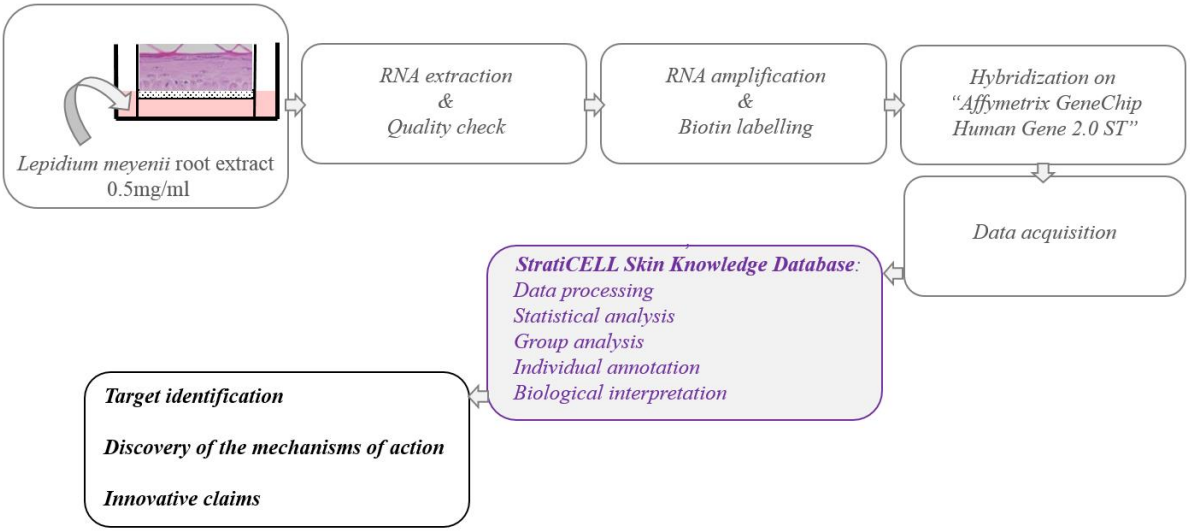
As a proof of concept, the present study aims to analyze the effects of a *Lepidium meyenii* root extract onto standard reconstructed human epidermis at the gene expression level. Full transcriptome profiling was selected for data acquisition.

METHODOLOGY

In vitro reconstituted 3D human epidermis (0.63 cm^2) with melanocytes phototype IV-V were cultivated in serum-free medium at the air-liquid interface for 14 days in a humidified incubator at 37°C with 5% CO_2. The *Lepidium meyenii* root extract was then applied in the culture medium for 24 hours at 0.5mg/ml (triplicates of culture). Total RNA was then extracted and sample integrity was analysed by spectrophotometry and capillary electrophoresis.

RNA amplification and biotin-labelling were performed prior to gene chip hybridization, in agreement with Affymetrix standard protocol. Hybridization was performed on Affymetrix GeneChip Human Gene 2.0 ST arrays.

The database was created on the basis of the literature and internal skin-specific knowledge/historical experiments, the general publicly available annotations, the mappings with public repositories and technological information (*i.e.* Affymetrix probes/targets mappings).
Group analysis was performed by taking into consideration only genes presenting expression variation ≥ 2, and with p values < 0.01 (hypergeometric test).
The overall protocol from product application to the data interpretation is illustrated below.



RESULTS AND DISCUSSION

Gene expression data were analysed and annotated using the database. The group analysis program/interface identified several statistically significant pathways. They are listed in the figure below. The number of genes in each group as well as the respective p-values is represented. Only statistically-significant themes and groups are represented. Data were filtered through a p-value < 0.01 for group analysis changes and a fold-change ≥ 2.

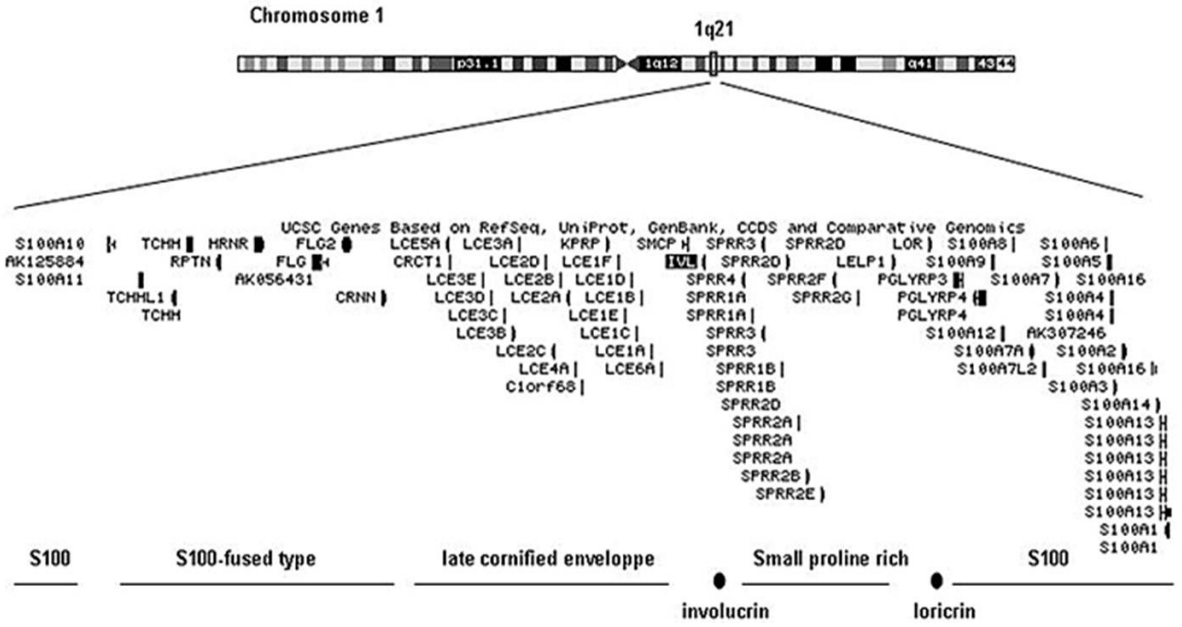
Themes	Groups	p-value	# Detected	# Members
Cell Proliferation	Cell Cycle & Proliferation	3.7e-2	1	35
Cytokines & Chemokines	Cytokines & Chemokines	1.1e-5	6	88
Epidermal Cohesion	Dermal-Epidermal Junction	4.8e-4	2	18
Epidermal Homeostasis	Epidermal Biology	1.3e-3	3	55
	Cornified Envelope Proteins	9.2e-6	3	16
	Differentiation Markers	5.5e-3	1	13
	Epidermal differentiation complex (EDC)	**2.2e-16**	**12**	**63**
Inflammation	Inflammation Response	8.2e-11	19	374
	NFkappaB Signalling	2.3e-7	9	132
Senescence & Ageing	Extracellular Matrix Components	1.6e-3	4	97
Skin Immune Response	Anti-microbial Defense, AMPs	0	19	173
	Dendritic & Antigen-presenting Cells	3.4e-2	2	82
	Toll-like Receptor Signalling	7.4e-5	5	81
	T-Cell Immune Response	1.8e-3	6	201
Stem Cell	Stem Cell & niche	1.4e-2	5	227
UV & Stress Response	p53 Signalling	5.9e-3	3	83
	Apoptosis	4.2e-3	8	364
	Osmotic Stress	6.9e-4	4	81
Wound Healing	Angiogenesis	6.1e-6	10	234
	Wound Healing	1.3e-4	10	328
	Remodelling Enzymes	8.8e-8	4	14

Among the groups identified and listed above, the group composed of the genes belonging to the epidermal differentiation complex demonstrated to be the most impressive, in terms of impacted genes. In the next section, we selected this group as an example, and will focus on these data to go deeper in the individual analysis of the differentiation genes.

Basically, differentiation of epidermal keratinocytes is based on specific program of gene expression changes, starting from the basal keratinocyte layer, and ending on the surface of the epidermis, in a process called desquamation.

A cytogenetic band of 2Mb, 1q21, contains ~60 genes encoding essential structural proteins and enzymes needed to ensure proper terminal differentiation of the keratinocytes and the formation of the cornified envelope (CE).

Most of these proteins belong to 4 families, *i.e*. the S100 family, the SPRRs for small proline-rich proteins, the late cornified envelope LCEs and SFTPs for S100-fused-type proteins. This set of genes is known as the Epidermal Differentiation Complex (EDC, cf. figure below) (Henry *et al*., 2012).



As an evidence of the involvement of the *Lepidium meyenii* root extract in epidermal differentiation and renewal, we identified 28 genes up-regulated by the *Lepidium meyenii* root extract that are part of the EDC (Epidermal Differentiation Complex) when data were filtered through a p-value < 0.05 for individual changes and a fold-change at least 1.3. They are listed in the table below.

Genes from the EDC that are regulated by the *Lepidium meyenii* root extract				
Symbol	**Name**	**Mapping**	**FC**	**p-value**
HRNR	Hornerin	1q21.3	9,51	6,76e-05
LCE3A	Late cornified envelope 3A	1q21.3	7,2	1,21e-04
LCE3C	Late cornified envelope 3C	1q21.3	7,17	3,21e-04
LCE3B	Late cornified envelope 3B	1q21.3	4,7	2,62e-03
S100A12	S100 calcium binding protein A12	1q21	3,96	2,91e-04
SPRR3	Small proline-rich protein 3	1q21-q22	3,73	2,95e-03
CRCT1	Cysteine-rich C-terminal 1	1q21	3,4	5,67e-03
SPRR2C	Small proline-rich protein 2C (pseudogene)	1q21-q22	2,89	8,39e-04
RPTN	Repetin	1q21.3	2,63	1,25e-03
S100A9	S100 calcium binding protein A9	1q21	2,19	1,06e-03
S100A7	S100 calcium binding protein A7	1q21	2,18	8,11e-03
SPRR2F	Small proline-rich protein 2F	1q21-q22	2,18	8,74e-03
PGLYRP4	Peptidoglycan recognition protein 4	1q21	2,07	1,45e-02
SPRR2B	Small proline-rich protein 2B	1q21-q22	2,07	5,67e-03
PGLYRP3	Peptidoglycan recognition protein 3	1q21	1,99	5,15e-03
SPRR2D	Small proline-rich protein 2D	1q21-q22	1,98	8,04e-03
SPRR1B	Small proline-rich protein 1B	1q21-q22	1,92	4,02e-03
LCE3E	Late cornified envelope 3E	1q21.3	1,82	7,03e-03
C1orf68	Chromosome 1 open reading frame 68	1q21.3	1,8	3,10e-03
LCE4A	Late cornified envelope 4A	1q21.3	1,64	4,67e-02
SPRR2A	Small proline-rich protein 2A	1q21-q22	1,58	3,11e-02
KPRP	Keratinocyte proline-rich protein	1q21.3	1,56	2,03e-02
LCE1F	Late cornified envelope 1F	1q21.3	1,52	4,40e-02
S100A8	S100 calcium binding protein A8	1q21	1,48	1,06e-02
LCE3D	Late cornified envelope 3D	1q21	1,43	3,73e-02
SPRR4	Small proline-rich protein 4	1q21.3	1,4	4,72e-02
SPRR1A	Small proline-rich protein 1A	1q21-q22	1,38	9,37e-04
SPRR2G	Small proline-rich protein 2G	1q21-q22	1,3	3,90e-02
S100A7L2	S100 calcium binding protein A7-like 2	1q21.3	0,76	4,33e-02
TCHH	Trichohyalin	1q21.3	0,55	1,95e-02

All these genes play a role in the epidermal differentiation process, and most of them are essential to the formation of the CE.

During this coordinated process of differentiation, cells regulate the expression of many genes, and undergo changes in metabolism and structure. At the end of their differentiation, keratinocytes of the granular layer are engaged in the process of cornification (Candi *et al*., 2005), which consists in the elimination of intracellular organelles, nuclear loss, aggregation of intermediate filaments and assembling of a rigid envelope in the periphery of the corneocytes, called the CE. The CE plays an essential role in the strength of the *stratum corneum* and water retention. These are held together by corneodesmosomes, and immersed in a lipid environment containing cholesterol, ceramides and fatty acids. The corneocytes and the lipids are schematically represented as a brick and mortar structure (Nemes and Steinert , 1999).

- The hornerin (HRNR) is a protein present in the cornified and granular layers of the epidermis. Recent works have shown that this protein is structurally closed to the profilaggrin (filaggrin precursor) which has a well characterized role in the terminal differentiation of keratinocytes, CE formation and epidermis moisturizing. Hornerin is localized in periphery of corneocytes and is crosslinked to other CE proteins through the action of transglutaminase-3 (Henry *et al*., 2012). Its function is to reinforce the CE and to contribute to mechanical resistance of the *stratum corneum*. A role in antimicrobial defense has also been proposed for some hornerin-derived peptides (Wu *et al*., 2009).

- The small proline-rich proteins (SPRR) family comprises four groups (SPRR1, -2, -3, and -4) that are expressed during late differentiation, in barrier-providing epidermis. Differential expression of SPRR proteins may modulate barrier activity over the surface of the skin through modulation of envelope biomechanical properties and in responsive to environmental stimuli and insult. Moreover, they have been proposed as reactive oxygen species quenchers, with an important function at the edges of wounds (Schäfer and Werner, 2011).

- The human LCE cluster encodes proteins related to SPRR proteins, *i.e*. the proteins are also CE precursors that have protein cross-linking functions similar to SPRR, the LCE cluster contains "groups" encoding conserved proteins, there is differential expression between group members, and there is specific expression in response to changed environmental conditions (Jackson *et al*., 2005).

- The S100 proteins are protein cross-linking components of the keratinocyte-cornified envelope. Based on biochemical evidence, differential expression, and association of an SPRR isoform with a particular CE morphology, it has been proposed that isoforms have distinct functions related to modification of CE properties at different anatomical sites or in response to environmental stresses, such as ultraviolet (UV) irradiation (Jackson *et al*., 2005).

In addition to the genes clustered within the EDC, the *Lepidium meyenii* root extract up-regulates 2 major genes coding for cross-linking enzymes, *i.e.* TGM1 and TGM5 (transglutaminases 1 and 5). Evidences indicate that TGM1 may be responsible for the attachment of some specific ceramides to the corneocytes envelope (Hitomi, 2005). TGM-5 is an enzyme that is responsible for cross-linking between proteins such as loricrin, involucrin and SPRR to form the CE.

Finally, it is important to notice that both AQP-3 and AQP-5 demonstrated to be up-regulated by the *Lepidium meyenii* root extract. The aquaporins (AQPs) are a family of membrane proteins that form water channels across cell membranes. Among skin AQPs, only AQP3 and AQP5 are strictly relevant to skin physiology and play thus important roles in epidermis hydration. AQP5 is believed to be a specific water channel. In contrast AQP3 is permeable both to water and glycerol and is the most abundant AQP in the epidermis (Boury-Jamot *et al*., 2006).

Some of the more relevant changes were confirmed by TaqMan qPCR.

CONCLUSIONS

The *Lepidium meyenii* root extract was applied on human reconstituted epidermis in order to investigate its effects at the gene expression level. Full genome DNA microarrays were used for data acquisition, and a new *Skin Knowledge Database* was used for data annotation, contextualization before interpretation and target/claim identification.

Among the major epidermal processes modulated by the *Lepidium meyenii* root extract, terminal differentiation of the keratinocyte was the most represented, with a bundle of evidence for a strengthening of the CE. The next steps will be to show that the data at the gene expression level are well correlated with protein abundance and functional effects.

By positive regulation of many genes associated with terminal differentiation of the epidermis, the extract could thus be effective to promote the most important function of the skin, the cohesion/barrier function of supra-basal layers of these tissues and the limitation of water loss, to maintain better hydration of the tissue.

In conclusion, this study proved the usefulness of gene expression profiling to identify novel claims and innovative targets in a cosmetic perspective. Moreover, the *Skin Knowledge Database* by StratiCELL demonstrated to be on scope and highly relevant for skin specific data analysis.

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