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# A Skin Extracellular Signaling Pathway via Intercellular Exosomes Highlighted by Genetic and Epigenetic Analysis Applied to a Natural Ingredient

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## INTRODUCTION

The term “inflammaging” explains the relation that exists between the aging process and inflammatory responses in individuals. This aging-linked chronic inflammation is mainly due to a functional decline in the immune system and cellular senescence process. With aging, pro-inflammatory mediators induce a positive feedback loop on immune cells activation and acquisition of senescent phenotype resulting in the accelerated aging [1]. Pro-inflammatory cytokines play an important function through activation of a key transcription factor, nuclear factor-kappa B (NF-κB), which activates inflammation [1]. Several activators of NF-κB exist, including the toll-like receptor pathway which is activated by toll-like receptor ligands like TNF-α. Pro-inflammatory cytokines recruit inflammation cells which activate dermal fibroblasts and induce secretion of proteases (matrix metalloproteinases, elastase) that degrade the extracellular matrix [2].

Intercellular communication is an essential hallmark of multicellular organisms. Communication between neighboring cells is mediated by cellular junctions, cellular adherence or secreted molecules such as hormones. In the last two decades, a supplementary mechanism for intercellular communication has emerged that involves intercellular transfer of extracellular vesicles [3]. Different kinds of cells release

vesicles of endosomal membrane origin called exosomes into the extracellular environment. Subsequently, these small vesicles are internalized in target cells through several mechanisms of interaction.

Exosomes are responsible for intercellular communication through the exchange of regulator factors secreted between neighboring and surrounding cells. Lipids and proteins are the main components of exosome membranes, but they also contain messenger RNAs (mRNAs), micro-RNAs (miRNAs) and other non-coding RNA

(RNanc) as well as diverse molecules involved in the formation, secretion and trafficking of exosomes [4]. The composition, biogenesis and secretion of exosomes are process regulated and influenced by environmental conditions. Indeed, after external stimuli, changes in exosomal protein and RNA content can influence responses of distant cells by inducing synthesis, protective or destructive signals [5].

Different cells use this communication as a means of delivering signaling molecules, sometimes called circulating miR-

## Abstract

*Skin aging, characterized at the cell level by structural and functional degradation, is linked to a chronic inflammation phenomenon also known as inflammaging. The aim of our study was to determine the link between inflammation and epidermis-dermis cross-talk, which declines with aging. Communication between the epidermis and dermis is an essential phenomenon that occurs via intercellular messengers, the exosomes. The signalosomes transport lipids, proteins and RNA molecules, including the micro non-coding RNA (miRNA) identified as mediators of cell functions, to target cells. Thus, these intercellular*

*signalosomes regulate the bioactivities of recipient cells via an epigenetic mechanism that dysfunctions with age. To investigate the inflammation signaling pathway between aged keratinocytes and fibroblasts, we designed a specific study model using the RT-qPCR method that allowed us to follow, analyze and understand the impact that exosomes have on cells. We then confirmed that exosomes extracted and isolated from keratinocytes directly influence fibroblast activity. In addition, we could show that a natural ingredient, sea silt extract, modified exosomal content rich in factors linked to the inflammaging phenomenon, inducing specific fibroblast biological responses.*

NAs. Micro-RNAs are post-transcriptional regulators of gene expression and play an important role in cellular function. In this way, exosomes regulate through epigenetic mechanisms biological activities of close or distant target cells. Given this ability to regulate simultaneously many targets, miRNAs are fundamental regulators in physiological conditions, such as in the aging process [6].

Crosstalk between cells inside a tissue or surrounding tissue constitutes an efficient defense strategy. With age, exosomal content is modified, leading to messages of protein degradations and pro-inflammatory cytokine production to target cells [7]. Some miRNAs are modulators of pathways related to the inflammation process and are called inflammamiRs [8]. For example, an increase in the circulating miR-21 level is a biomarker of inflammaging and also linked to the NF- $\kappa$ B pathway [9].

Starting from the premise that deleterious exchanges between epidermis and dermis could lead to dysfunction of the dermis, there was a major interest in targeting our research on understanding the exosome-mediated inflammation communication between the epidermis and dermis. The aim of this study was to show the role of exosomes released by 40-year-old normal human epidermal keratinocytes in the transmission of inflammatory factors and the negative regulation of the extracellular matrix and then prove that an anti-inflammatory natural ingredient, by modulating miRNAs and gene expression, was able to correct the message sent by the keratinocyte to the dermis *via* the exosomes.

## EXPERIMENTAL

### Primary keratinocyte culture

40-year-old normal adult human primary epidermal keratinocytes (nHEK) provided by ATCC (Molsheim, France) were maintained in an incubator equilibrated with  $5 \pm 1.5\%$  CO<sub>2</sub> at  $37 \pm 2^\circ\text{C}$  in a specific Dermal Cell Basal Medium (ATCC) supplemented with keratinocyte growth kit components containing 0.4% of bovine pituitary extract, 0.5 ng/mL of rh TGF- $\alpha$ , 6 mM of L-glutamine, 100 ng/mL of hy-

drocortisone hemisuccinate, 5  $\mu\text{g}/\text{mL}$  of insulin, 1.0  $\mu\text{M}$  of epinephrine and 5  $\mu\text{g}/\text{mL}$  of apo-transferrin (ATCC).

### Exosome purification and quantification of keratinocytes

Keratinocytes were seeded in T175 flasks (BD Falcon) until cells reached 80% of confluency. Then the medium was replaced with fresh culture media and cells were incubated 24 hours before exosome extraction. Next the supernatants were collected and cell debris was removed by centrifugation for 30 min at 3500 g and  $4^\circ\text{C}$ . The culture media were then concentrated on a column filter (Amicon Ultra-15 100K centrifugal filter devices, Merck-Millipore, Fontenay-sous-Bois, France). Exosomes from the concentrated cell culture media were isolated using the specific kit EXOPrep (Hansabiomed, Tallinn, Estonia) according to the manufacturer's instructions. Pelleted exosomes were resuspended in sterile PBS 1X. Exosomes in the suspension were quantified using the specific kit EXOCET Exosome Quantitation Assay kit (System Biosciences, Palo Alto, California, USA) according to the manufacturer's instructions. It is an enzymatic colorimetric assay measuring the absorbance at 405 nm of esterase activity known to be within exosomes. The exosome proteins were extracted using RIPA buffer (Sigma, Saint-Quentin Fallavier, France) and quantified with a bicinchoninic acid (BCA) protein assay kit (Pierce, Saint-Aubin, France).

### Exosomal stimulation of primary fibroblasts

40-year-old normal adult human primary dermal fibroblasts (nHDF) provided by ATCC (Molsheim, France) were maintained in an incubator equilibrated with  $5 \pm 1.5\%$  CO<sub>2</sub> at  $37 \pm 2^\circ\text{C}$  in a specific "fibroblast basal medial" medium (ATCC) supplemented with fibroblast growth kit (ATCC) components containing 2% fetal bovine serum (FBS), 7.5 mM L-glutamine, 1  $\mu\text{g}/\text{mL}$  hydrocortisone, hemisuccinate, 5 ng/mL rh FGF- $\beta$ , 5  $\mu\text{g}/\text{mL}$  rh insulin, and 50  $\mu\text{g}/\text{mL}$  ascorbic acid. To test the ability of exosomes to stimulate primary fibroblasts, fibroblasts were seeded on 6-well plates at a 120,000 cells per well density 24 hours prior to exosome stimulation. For the stimulation step, culture media was

removed and replaced by fresh medium with exosome quantities corresponding to 5  $\mu\text{g}$  of exosomal proteins. Fibroblasts were next incubated 24 hours at  $37^\circ\text{C}$  and  $5\%$  CO<sub>2</sub>. For the negative control, exosomes were substituted with PBS in cell culture media. Three biological replicates were prepared and all experimental assays (RT-qPCR) were performed in duplicate for each biological replicate. As a key protein modulated by exosomal stimulation, matrix metalloproteinase (MMP-1) expression was measured with the RT-qPCR method in fibroblasts.

### Extraction and expression of miRNA and mRNA by the RT-qPCR method

Total RNA and miRNAs from keratinocytes, exosomes and stimulated fibroblasts were extracted and purified using a miRCURY RNA Isolation Kit (Exiqon, Vedbaek, Denmark) following the manufacturer's instructions. Quality control and total RNA quantification were performed using the Agilent RNA Nano kit and Analysis Agilent 2100 bioanalyzer. For micro-RNA quantification, total RNA was reverse-transcribed with the Universal cDNA Synthesis Kit II (Exiqon) according to the manufacturer's instructions. Reverse transcription reactions were diluted and a fraction of diluted cDNA was used for each quantitative PCR. Quantitative PCR was performed with a Exilent SYBR Green master mix kit (Exiqon) using the Stratagene MX3005P system (Agilent Technologies, Les Ulis, France) or the CFX connect system (Biorad, Marnes-la-Coquette, France). The results were normalized to RNU1A expression. Reference and expression levels of miRNA were calculated using the  $2^{-\Delta\Delta\text{Ct}}$  method. For mRNA target quantification, total RNA was reverse-transcribed with the Superscript VILO cDNA Synthesis Kit (ThermoFisher, Saint-Aubin, France) according to the manufacturer's instructions. Quantitative PCR was performed with a Platinum Quantitative PCR SuperMix-UDG Kit (Invitrogen, Saint-Aubin, France) using the Stratagene MX3005P system (Agilent Technologies) or the CFX connect system (Biorad). The results were normalized to endogenous control 18S expression and the expression level of the mRNA target was calculated using the  $2^{-\Delta\Delta\text{Ct}}$  method.

### Sea silt extract

The natural ingredient studied is a marine microorganism extract which contains fatty acids and sterols. To evaluate this natural ingredient *in vitro*, nHEK cells were treated with 50 ppm of the sea silt extract for 24 hours.

### Statistical Analysis

The results presented are the means  $\pm$  SEM. Differences between groups were assessed by means of Student's unpaired t-test. The significance level was set at  $*p < 0.05$ .

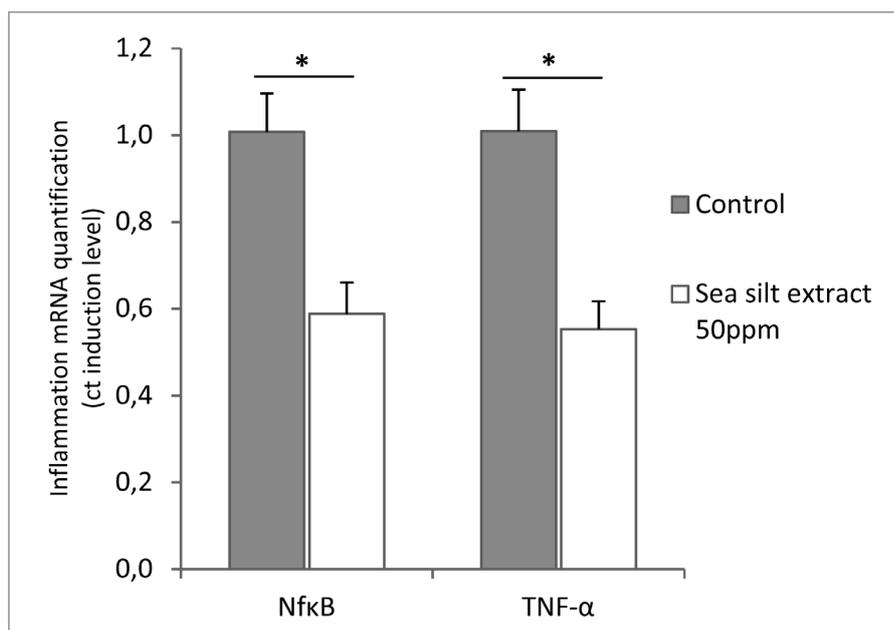
## RESULTS

### Direct ingredient impact on the inflammatory state of keratinocytes

During cutaneous aging, cells are exposed to inflammatory factors that cause deleterious damage to cellular function. We studied the effect of the sea silt extract on the inflammation process in a keratinocyte culture. The level of two pro-inflammatory agents, TNF- $\alpha$  and NF- $\kappa$ B, was measured using RT-qPCR (Figure 1). Under the experimental conditions, the natural ingredient seemed to reduce inflammation in epidermal cells *via* a significant decrease of 46% TNF- $\alpha$  and 42% NF- $\kappa$ B ( $*p < 0.05$ ) expression, respectively.

### Epigenetic modulation of inflammation from epidermis to dermis

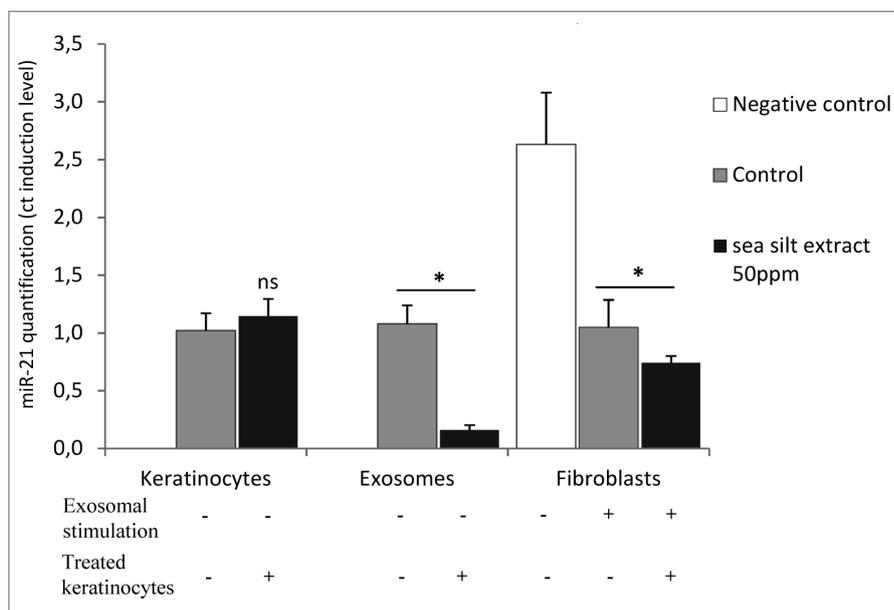
We validated the experimental protocol in which 5  $\mu$ g of exosomes extracted at 24 h of a keratinocyte culture have a potent capacity of stimulation after 24 h of incubation in fibroblasts. In this condition, MMP-1 expression, known as an exosomal stimulation biomarker, was increased 14 times compared with untreated fibroblasts (data not shown). This confirmed that such exosomes were able to induce an extracellular matrix degradation message in fibroblasts. In this model, we tested a natural ingredient to evaluate its potential to modulate and regulate genes and miRNAs involved in cellular inflammaging. Levels of specific miRNAs (miR-21, miR29-a et miR-29b) were measured using the RT-qPCR method in keratinocytes, exosomes and fibroblasts (Figure 2). Those miRs in keratinocytes treated with sea silt extract were decreased by up to 52%. The exception was miR21, which showed no significant

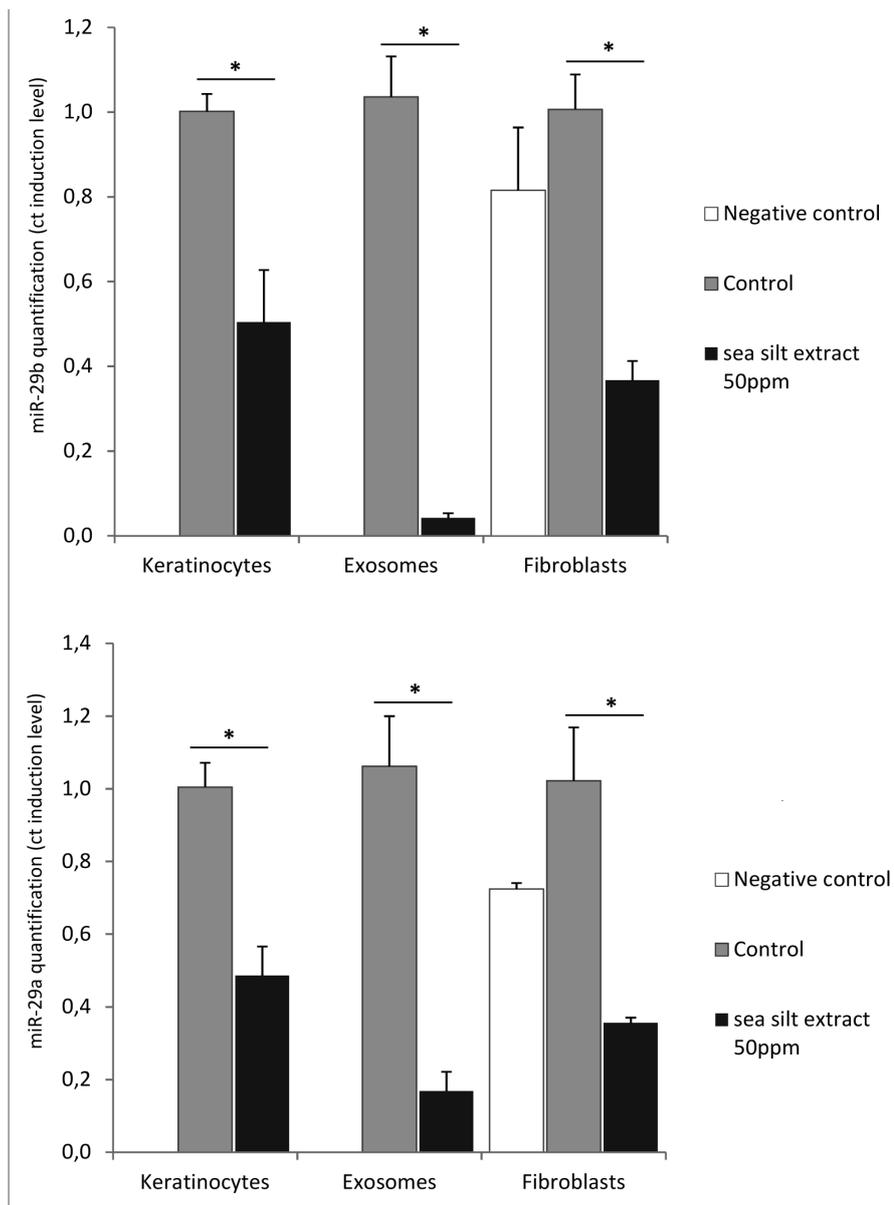


**Figure 1** Effect of sea silt extract on the level of mRNA related to inflammation (NfκB and TNF- $\alpha$ ) in keratinocytes using RT-qPCR. Control nHEK (gray profile) and nHEK treated with sea silt extract (white profile) were cultured according to the validated experimental protocol. Target mRNAs were purified and then quantified using RT-qPCR. mRNA levels are expressed as induced arbitrary unit relative to the control ( $\Delta$ ct). Endogenous control =  $\beta$ -18S mRNA expression; significant p-value:  $*p < 0.05$  versus control (n=3); Student's t-test).

difference from the control condition. The results also revealed a significant decrease in miR21 and miR29a-b in exosomes of more than 90% ( $*p < 0.05$ ). These exosomes were used to stimulate fibroblasts. The unstimulated fibroblasts (negative control) showed a decrease in miR29a-b expression compared with stimulated fibroblasts (positive control), suggesting

that the exosomal stimulation induced a positive modulation of the expression of these miRs (Figure 2). The levels of miR29a and -b and miR21 in fibroblasts stimulated with exosomes from keratinocytes treated with sea silt extract were downregulated by up to 67% compared with fibroblasts stimulated with exosomes from untreated keratinocytes (positive control) ( $*p < 0.05$ ).





**Figure 2** Measurement of miR-21, miR-29a and -29b levels in keratinocytes, exosomes and fibroblasts. Control cells (gray profile) and cells treated with sea silt extract 50 ppm (black profile) were cultured according to the validated experimental protocol. nHDF that were not stimulated with exosomes were cultured as the negative control (white profile) and nHDF stimulated with exosomes of untreated keratinocytes were cultured as the positive control (gray profile). Micro-RNAs of keratinocytes, exosomes and fibroblasts were purified and then quantified using RT-qPCR. Results are expressed as the induced arbitrary unit relative to the control ( $\Delta$ ct) (positive control (gray) for fibroblasts, control (gray) for keratinocytes and exosomes). Endogenous control = RNU1A expression; significant p-value: \* $p < 0.05$  versus control ( $n=3$ ); ns: not significant (Student's t-test).

### miRNA's genetic regulation from epidermis to dermis

To understand the miRNAs regulation in cutaneous cells, we focused our research on the TGF- $\beta$ /smad pathway which regulates miRNAs. Thus, mRNA of TGF- $\beta$ , TGF- $\beta$ RII and smad3 were quantified in keratinocytes and fibroblasts (**Figure 3**).

The results showed a significant increase in TGF- $\beta$  and smad3 expression of 42% and 50%, respectively in keratinocytes treated with sea silt extract compared with untreated keratinocytes (control). We also observed a significant increase in TGF- $\beta$ RII and smad3 of 3.4-fold and 2.5-fold, respectively, in fibroblasts stimu-

lated by exosomes of keratinocytes treated with sea silt extract compared with fibroblasts stimulated by exosomes of untreated keratinocytes (control) ( $p < 0.05$ ).

### Indirect effect of the ingredient on ECM synthesis by activated fibroblasts

To evaluate the effect of sea silt extract on fibroblast response after exosomal stimulation, the levels of mRNA COL (collagen), ELN (elastin) and FBN1 (fibrillin) were measured using RT-qPCR (**Figure 4**). In physiological conditions, aging skin cells have a basal level of mRNA related to the ECM components. This level is not modified after exosomal stimulation. However, fibroblasts that were stimulated with exosomes extracted from keratinocytes treated with sea silt extract showed a significant increase in COL, ELN and FBN1 expression of up to 2.6, 4 and 2 times that of cells stimulated by exosomes of untreated keratinocytes, respectively ( $p < 0.05$ ). These results demonstrate that exosomes extracted from treated keratinocytes deliver to fibroblasts a final message of extracellular matrix restructuring through the synthesis of its main components.

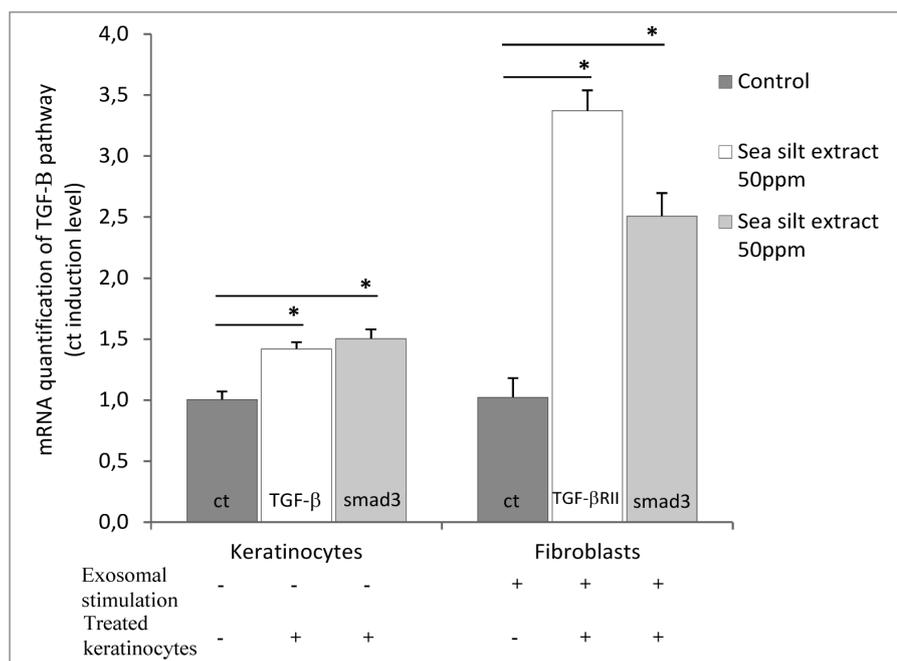
## DISCUSSION

### Effect of sea silt extract on epidermis inflammation

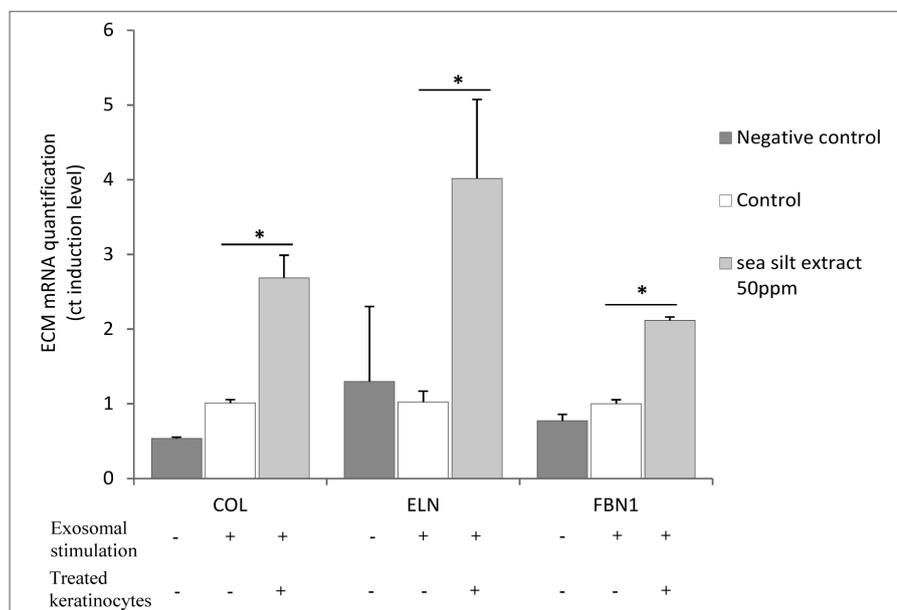
NF- $\kappa$ B and TNF- $\alpha$  are pro-inflammatory modulators in the signaling toll-like receptor (TLR) pathway, which is a key pathway in the inflammaging phenomenon. TLR receptors, mainly involved in response to a pathogen, also play a role in recognition of endogenous ligands like TNF- $\alpha$  cytokine, which activates the pathway and can be the source of chronic inflammation. Sea silt extract contains specific sterols that have an inhibitory effect on TNF- $\alpha$ , and conjugated fatty acids which present anti-inflammatory effects. Thus, we hypothesis that the sea silt extract decreases activation of the TLR pathway through a reduction of TNF- $\alpha$  binding and the transcriptional activity of NF- $\kappa$ B.

### Transmission of a message between epidermis and dermis

The study allowed the establishment of an innovative experimental method for targeted evaluation of exosome-mediated in-



**Figure 3** Quantitative analysis of mRNA related to the TGF-β/smads pathway (TGF-β, TGF-βRII, smad3) in keratinocytes and fibroblasts using RT-qPCR. The control (dark gray profile) and cells treated with sea silt extract 50 ppm (other profile) were cultured according to the validated protocol (keratinocyte control= untreated keratinocytes, fibroblast control = fibroblasts stimulated with exosomes of untreated keratinocytes). mRNA of TGF-β, TGF-βRII, smad3 of keratinocytes and fibroblasts were purified and then quantified using RT-qPCR. mRNA levels are expressed as the induced normalized arbitrary unit relative to control ( $\Delta$ ct); endogenous control = 18S mRNA expression; significant p-value: \* $p < 0.05$  versus control (n=3); Student's t-test).



**Figure 4** Effect of sea silt extract on extracellular matrix mRNA (COL, ELN, FBN1) in fibroblasts. Control nHDF untreated and unstimulated (gray profile), nHDF stimulated by exosomes from untreated keratinocytes (white profile) or nHDF treated with sea silt extract (light gray profile) were cultured according to the validated protocol. mRNA of fibroblasts were purified and then quantified using RT-qPCR. mRNA levels are expressed as the induced arbitrary unit (and normalized) relative to control ( $\Delta$ ct); endogenous control = 18S mRNA expression; significant p-value: \* $p < 0.05$  versus control (n=3); Student's t-test).

tercellular communication that we used to study the efficacy of a natural ingredient. The results first provided information on the physiological message sent between aging skin cells through exosomes. These exosomes from keratinocytes seem to relay a message to fibroblasts correlated with a pro-inflammatory state and protein degradation through MMP-1 and miR29a-b expression.

Through its direct interaction with keratinocytes, sea silt extract caused a modification in exosomal content. This content was discharged into the fibroblast cytoplasm and induced a specific response similar to that of the keratinocyte, i.e. a repression of specific miRs and an increase in TGF-β/smads pathway activation. This suggests that the decrease in NF-κB expression in keratinocytes induced a downregulation of miR21 [9] and finally an overexpression of the TGF-β pathway. As a matter of fact, studies in different cellular model revealed a mechanism in which the miR-21 negatively modulates this pathway by targeting TGF-βRII receptor. The binding of TGFβ ligand with its receptor TGFβRII/TGFβRI induces different signaling cascades involving transcription factor smad3.

The rise of TGF-βRII could increase the availability of TGF-β binding and potentially activate its signaling pathway. This activation triggers nuclear translocation of smad complex and facilitates the binding of this complex to DNA sequences and consequently may regulate genes and miRNAs expression. For example, biogenesis of miR-29 is repressed by the smad3 complex [10]. Based on these data from the literature and our experimental results, we concluded that miR-21 repression via the TLR pathway induced activation of the TGF-β/smads pathway and that miR-29 is repressed in keratinocytes and fibroblasts by this activation.

#### Reading by fibroblasts of the message derived from keratinocytes

Exosomal miR-29a and miR-21 have the ability to act as ligands by binding the TLR7 and TLR8 located in endosomes. This interaction activates the conventional signaling cascade of the TLR pathway and induces pro-inflammatory cytokine pro-

duction [10]. Action mechanisms of these miRs seem to be involved in inflammatory communication between keratinocyte and fibroblast. These different elements confirm our hypothesis that exosomes communicate an inflammation regulation message to dermal cells. These results also show that the communication network involving exosomes and their content is modified by the action of the natural extract directly on keratinocytes. The TGF- $\beta$  pathway is strongly involved in regulation of the synthesis and degradation of the extracellular matrix through positive regulation of COL expression. In parallel, miR-29a/b represses genes involved in COL expression [11]. Negative regulation of these miRs by the TGF- $\beta$ /smad3 pathway helps to improve collagen expression, while the reduction in miR-21 is also evidence of improved cellular function. Our results confirm an impact of sea silt extract on extracellular matrix synthesis thanks to the positive regulation of the TGF $\beta$  pathway and the negative regulation of miR-21 and miR-29.

## CONCLUSION

Cellular aging is characterized by chronic inflammation caused by immune system dysfunction, acquisition of cellular senescence and deregulation of epigenetic mechanisms. Exosome-mediated communication was studied and we could confirm the link between inflammation and cutaneous aging. This study showed the role of exosomes in transmission of inflammatory factors and in regulation of the extracellular matrix. Investigations showed that by regulating a specific metabolic pathway involving (epi)genetic modulations an anti-inflammatory natural ingredient can correct the message sent by keratinocytes to the dermis through acti-

vated exosomes. Transmission of the message induced inflammation regulation and ultimately restructuring of the extracellular matrix. In conclusion, the observation that these messengers, however small, are able to induce key reactions in the biological process linked to inflammaging offers a very interesting perspective for future studies on skin aging.

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