Technical Report

Product

REPROAGE™ *peptide*

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1

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Epigenetics: approaching skin care beyond the paradigm

Previously, the established view in biology was that every process in the cell was determined by the information written in the DNA sequence of genes. This changed along the past decades with the discovery of epigenetics.

Now, it is well known that there is a set of **molecular mechanisms regulating gene expression**, turning it on or off, independently of the genetic sequence of those genes. These processes are called **epigenetic** because they work above the genetic information. These molecular modifications can be transmitted to daughter cells and play an important part in various physiological functions, determining how cells behave [1, 2]. Epigenetic changes can occur with cellular **aging** and due to **external factors** (chemicals, diet or exercise) [3].

At the molecular level, epigenetic mechanisms are very diverse and include DNA methylation, modifications to histones, chromatin conformation and non-coding RNAs. **Non-coding RNAs** are transcribed from the cell genome, but they are not translated into proteins [4]. Among the most studied non-coding RNAs are the **microRNAs** (or miRNAs).

miRNAs are short (20–23 nucleotides) single-stranded RNA molecules and they **target specific mRNAs to silence their expression** through post-transcriptional interference [4, 5]. In this process, the miRNA recognizes and binds to target regions in the mRNA by complementarity between their sequences. This represses the mRNA either by preventing its translation into the corresponding protein or via initiating its degradation (figure 1) [5].

More than 2,500 miRNAs have been identified in human cells and each may target many different mRNAs, resulting in complex regulatory networks [4].

miRNAs are key regulators of genes involved in **several biological processes** including development, metabolism, apoptosis and senescence, cellular protection and detoxification and stem cell renewal [3, 6]. In the skin, they are important for processes such as **epidermal stratification and regeneration** [7]. Epigenetics knowledge can give us the possibility to control certain processes that tend to become dysfunctional, without affecting the DNA, opening new opportunities to develop a more efficacious skin care.

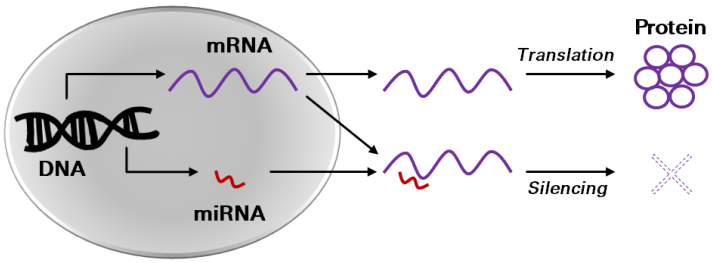


Fig. 1. Mechanism of gene function regulation by miRNAs.

Epigenetics, an alternative and powerful way to control biological processes, augur great potential for skin care.

Epidermal stem cells as drivers of skin regeneration

The epidermis is one of the most active tissues in terms of regeneration. It is organized into four stratified layers of keratinocytes at distinct maturation stages. From bottom to top, these are the *stratum basale* (germinal or basal layer), the *stratum spinosum*, the *stratum granulosum*, and the *stratum corneum*.

Keratinocytes move outward through all the epidermal layers while undergoing morphological and functional changes as part of differentiation [8]. At the top of the stratum corneum, they are continuously shed and therefore new differentiated cells must be generated throughout life in order to maintain the integrity of the epidermis. The **regenerative capacity** of the epidermis relies on the cells located at the **basal layer**, which consist of epidermal stem cells and transit amplifying (TA) cells, also known as **progenitor cells**.

Stem cells are unspecialized cells that are capable of dividing and renewing themselves for long periods (months to years). They undergo divisions to generate **TA cells**, which populate most of the basal layer. These are undifferentiated cells with a limited proliferative potential [9]. They undergo defined rounds of proliferation and eventually start the process of **differentiation into keratinocytes** (specialized cells) that start moving up through the corresponding epidermal layers as they mature.

The **regenerative potential** and overall function of the epidermis is **modified with age** [10]. The function of progenitor cells is impaired, as these cells slow down their cycle and undergo significantly fewer divisions, reducing the generation of functional keratinocytes and thus the efficiency of epidermal renewal [11, 12]. This **reduced turnover** of the epidermis results in the **development of aging signs**: the skin suffers a progressive decline in thickness and becomes loose, dry and prone to wrinkles, acquiring also an irregular pigmentation and losing radiance [13].

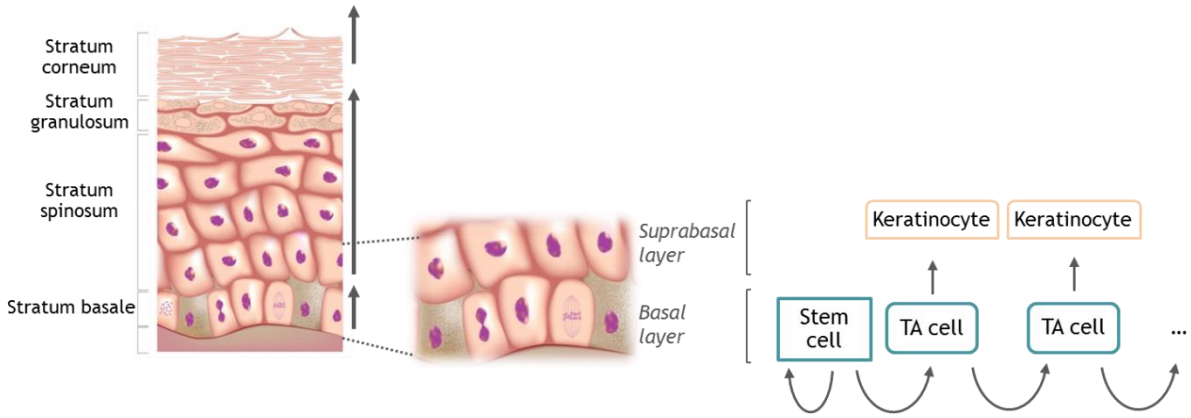


Fig. 2. Cells in the basal layer of the epidermis and maintenance of the progenitor cells pool.

Basal cells maintain the epidermal homeostasis, being key for skin rejuvenation.

Control of progenitor cells behavior and skin aging

The self-renewal abilities of basal cells are maintained by a regulatory network of genes that includes the **transcription factors OCT4**, **SOX2**, and **KLF4**, which are **stem cell markers**. These are key in controlling a wide range of downstream genes, promoting the expression of basal cell specific proteins and the maintenance of proliferation [14].

In addition to the above mentioned genes, there is an additional level of regulation provided by microRNAs. **microRNA-145** (miR-145) is important because it directly targets OCT4, SOX2 and KLF4. This miRNA represses the activity of the three stem cell transcription factors and as a result inhibits cellular self-renewal [14].

miR-145 levels are relatively low in basal cells and **increase to high levels during differentiation**, with a concomitant decrease of the stem cell transcription factors. Conversely, it is known that the inhibition of the miR-145 activates the stem cell factors that induce the cells self-renewal [14].

When progenitor cells in the basal layer increase the expression of miR-145 and lose regenerative properties, they begin to differentiate into keratinocytes and migrate towards the epidermal surface. Transition from the basal to the suprabasal layers involves many functional and molecular modifications and is characterized by a profound **change in the expression of characteristic cell markers**, including keratins and transcription factors [15].

Keratins 5 and **14** (K5 and K14) are strongly expressed in the undifferentiated cells of the basal layer, and they may have a role in maintenance of the regenerative potential [16]. During the differentiation process, expression of these proteins decreases progressively, while expression of suprabasal keratins (K1 and **K10**) starts to increase [15, 17].

The **transcription factor p63** is highly expressed in basal cells of the epidermis, where it is required to maintain the highly regenerative potential of these cells. It is down-regulated once the cells become suprabasal [15, 18].

Regulation of basal cells behavior **can become dysfunctional** with aging, resulting in a characteristic loss of vitality, with the consequent worsening in skin properties and appearance. Understanding the mechanisms of regulation through miR-145 and the stem cell factors in epidermal cells can provide a chance to **maintain** for longer or **reactivate** their **regenerative properties** and obtain **anti-aging** and **rejuvenating** benefits.

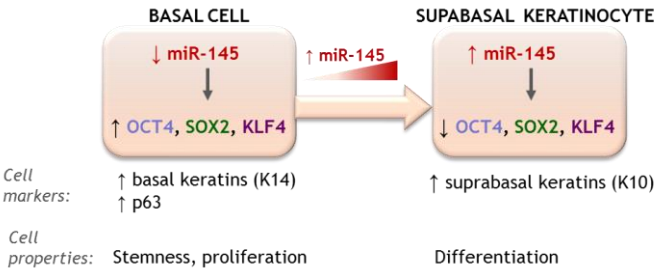


Fig. 3. Regulation of basal cell fate by miR-145.

miR-145 is a repressor of the factors involved in self-renewing properties of the epidermis.

REPROAGE™ *peptide*, reprogramming the skin for a youthful appearance

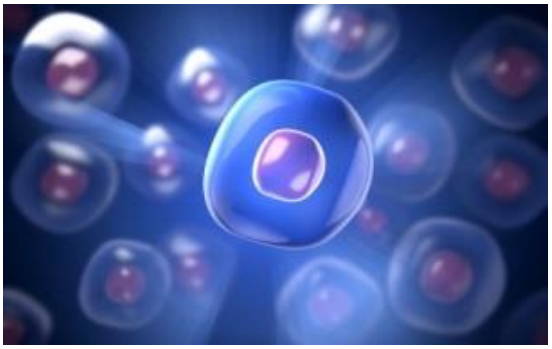
REPROAGE™ *peptide* is a cosmetic active ingredient inspired by epigenetics to help skin regeneration properties last longer for an improved appearance.

The peptide has proven to decrease the levels of **miR-145** in epidermal cells. Additionally, the **factors related to stemness,** which are repressed by this microRNA, were found to be consistently induced in the cells.

In epidermal models, the treatment with the peptide modulated the amount of specific markers of basal cells and suprabasal differentiated keratinocytes, suggesting a **reprogramming of the cells** in favor of the basal phenotype. This may indicate a reactivation of the pool of epidermal progenitors and the **potential for regeneration**.

As demonstrated by means of *in vivo* study, the application of a cream containing REPROAGE™ *peptide* was associated with a faster epidermal **self-renewal**, which could be a consequence of reactivated basal cells. The skin regenerated itself at a rate **equivalent to that of more than a decade younger skin** in volunteers of different age ranges.

In addition, an improvement of facial **skin appearance** was observed after treatment with the active ingredient, with increased **smoothness** and **radiance**.



REPROAGE™ *peptide* may help rejuvenate the epidermis and improve skin complexion.

In vitro efficacy

MODULATION OF MIR-145

The evaluation of the inhibitory properties of the peptide on the expression of miR-145 was performed using a direct miRNA quantification test.

Human keratinocytes were incubated for 24 hours with 10 µg/mL REPROAGE™ *peptide*, while non-treated cells were used as a control. After the treatment, the cells were lysed to release intracellular contents.

miR-145 was detected and quantified by means of a hybridization-based assay. In this test, the microRNA is hybridized to specific probes and the signal is detected by luminescence. Relative miR-145 levels were calculated with respect to control.

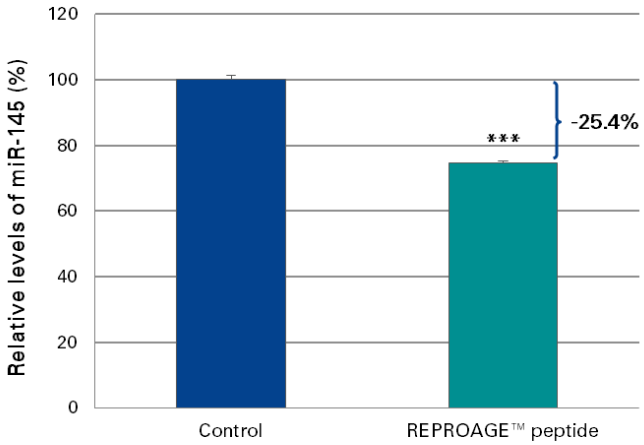


Fig. 4. Relative miRNA145 expression after incubation with the peptide (vs control: ***p<0.001).

After treating the cells with REPROAGE™ *peptide*, there was a statistically significant **decrease** in the **quantity of miR-145**.

Levels of the epigenetic regulator of epidermal cells fate were reduced.

INDUCTION OF STEM CELL TRANSCRIPTION FACTORS

Given the inhibition of miR-145 expression by REPROAGE™ *peptide*, changes in the levels of the stem cell markers that are modulated by this microRNA were studied.

HEKa were incubated with for 48 hours with 1 or 5 µg/mL REPROAGE™ *peptide*, while non-treated cells were used as a control.

Then, the cells were lysed and the gene expression profiles of stem cell markers SOX2, OCT4 and KLF4 were quantified by means of RT-PCR arrays.

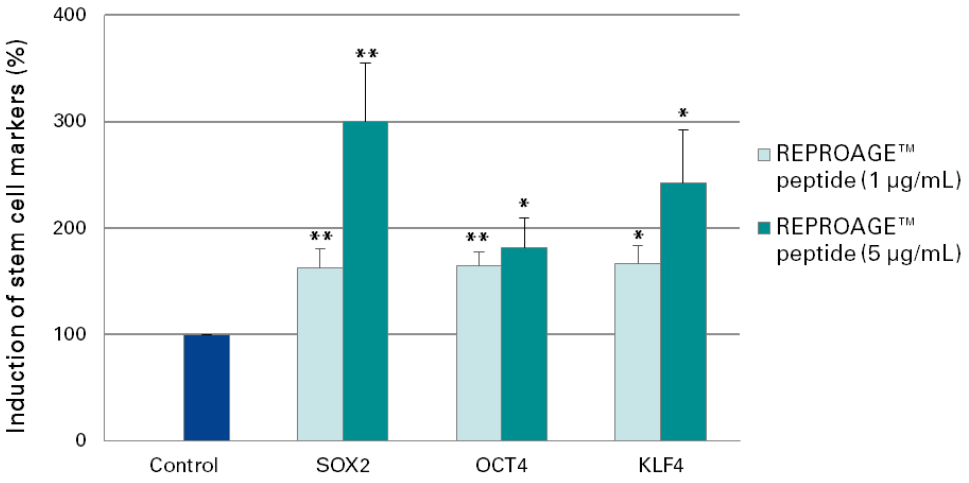


Fig. 5. Level of gene expression of the three transcription factors (vs control: *p<0.05; **p<0.01).

Induction of SOX2, OCT4 and KLF4 could suggest **cellular reprogramming**, with the gain of self-renewal properties.

Increase of factors essential for the maintenance of progenitor cells.

CHANGES IN KERATIN EXPRESSION PROFILE

The induction of changes in the expression of basal and suprabasal keratins was studied in keratinocytes during their differentiation, which was induced by increasing the calcium concentration in the medium.

HEKa were incubated with 1 or 5 µg/mL REPROAGE™ *peptide* in medium containing 1 mM $CaCl_2$. Cells incubated in medium alone were used as a basal control (non-differentiated cells). Cells incubated with 1 mM $CaCl_2$ medium were used as positive control of differentiation.

After 48 hours incubation, cells were lysed and their keratin expression profiles were analyzed by means of RT-PCR arrays.

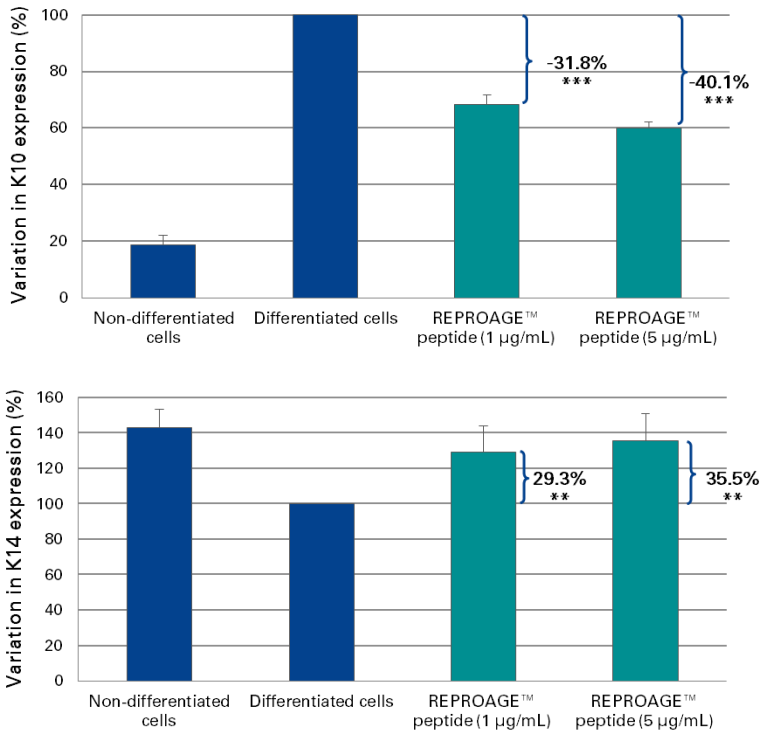


Fig. 6. Expression levels of suprabasal (K10) and basal (K14) keratins (vs differentiated cells control: **p<0.01; ***p<0.001).

REPROAGE™ *peptide* was found to **decrease gene expression** of suprabasal **K10** and **increase** that of basal **K14**.

Modulation of markers related to basal and suprabasal cells.

REPROGRAMMING OF EPIDERMAL CELLS

A skin model was used to verify changes in suprabasal and basal keratin proteins after treatment with REPROAGE™ *peptide*.

Reconstructed human epidermal (RHE) tissues were treated with 10 µg/mL REPROAGE™ *peptide* or with medium alone (control).

After 48 hours, proteins K10 and K14 were detected in the RHE tissues by means of immunohistochemistry and the level of each protein was quantified from fluorescence microscopy images.

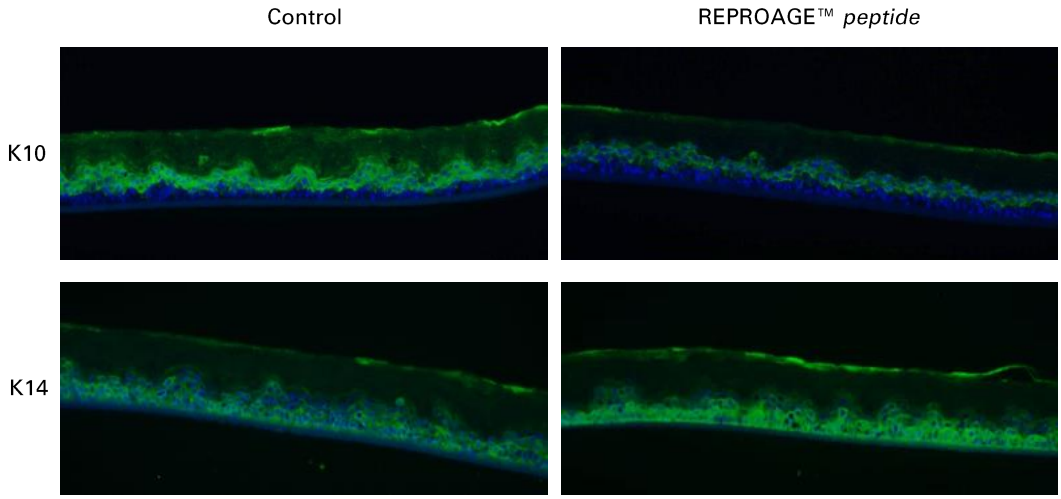


Fig. 7. Epidermal tissues showing K10 and K14 in green (nuclei are colored in blue).

The treatment **decreased K10** by 71.3% in the suprabasal layer and **increased K14** by 325.8% in the basal layer, both changes being statistically significant (***p<0.001).

Stimulation of the basal layer, formed by the progenitor and stem cells.

REVITALIZATION OF THE PROGENITOR CELLS POOL

In this study, the active ingredient was validated for its ability to favour the activation of the cells in the basal layer. p63 is specifically expressed in the nuclei of cells that are either proliferating or possess the ability to multiply.

RHE tissues were treated with 10 µg/mL REPROAGE™ *peptide* or medium alone (control) for 24 hours.

Then, protein p63 was detected by immunohistochemistry and the levels in each condition were quantified from fluorescence microscopy images.

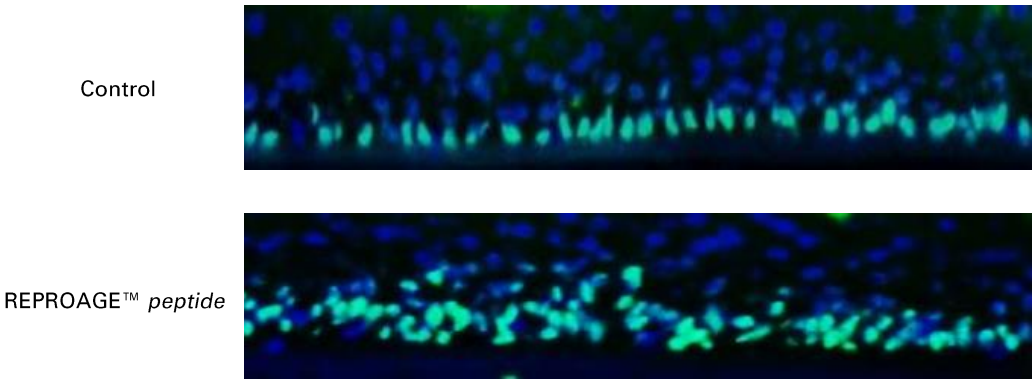


Fig. 8. Staining of epidermal models with p63 in green (cell nuclei are marked in blue).

The level of **p63** in basal epidermis was enhanced by **110.9%** (****p<0.0001), which suggests the activation on of the pool of epidermal progenitors.

Increased p63 may indicate a greater regenerative potential in the epidermis.

In vivo efficacy

RENEWAL AND SMOOTHING OF THE SKIN SURFACE

A clinical test was performed in order to measure the ability of the epidermis to renew itself and also changes in skin roughness after the application of a cream containing 2% REPROAGE™ *peptide solution*. The study was carried out in two groups of female volunteers of different ages: 20 subjects aged 35-40 years old and 20 aged 50-55 years old.

Renewal of the stratum corneum

The assessment of the epidermal renewal was done in forearm skin by means of dihydroxyacetone (DHA) staining.

Before the first product application, the skin was stained with DHA. Then, volunteers applied, twice a day, a cream containing 2% REPROAGE™ *peptide solution*, a placebo cream or no treatment. Color change was assessed by means of spectrophotometry and finally, the stratum corneum turnover time (SCTT) was calculated for each condition.

The basal SCTT of a younger group of volunteers (18-25 years old) was also measured and used to obtain a correlation between SCTT and age from the 3 groups of volunteers and interpolate the years of rejuvenation after each treatment.

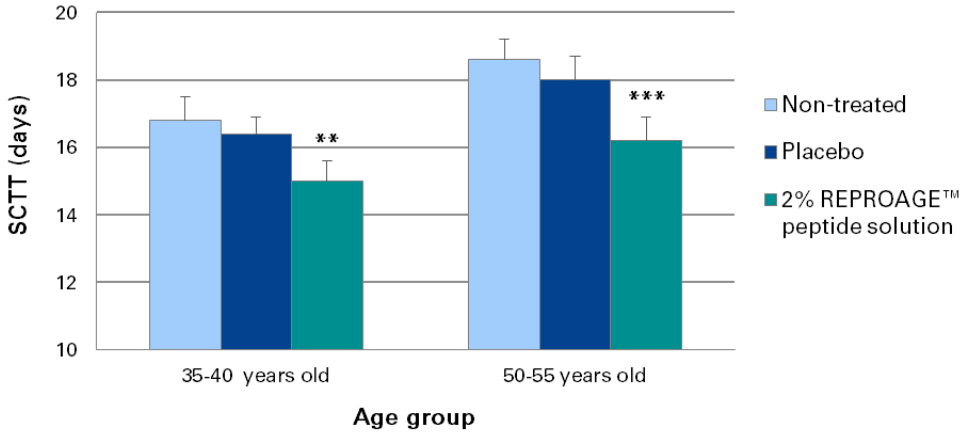


Fig.9. Time for self-renewal in the epidermis (vs non-treated skin: **p<0.01; **p<0.001; vs placebo: *p<0.05).

There was a significant decrease in the SCTT, also compared to placebo, **reducing differences between age groups**.

Revitalization of the skin, with an accelerated cell renewal.

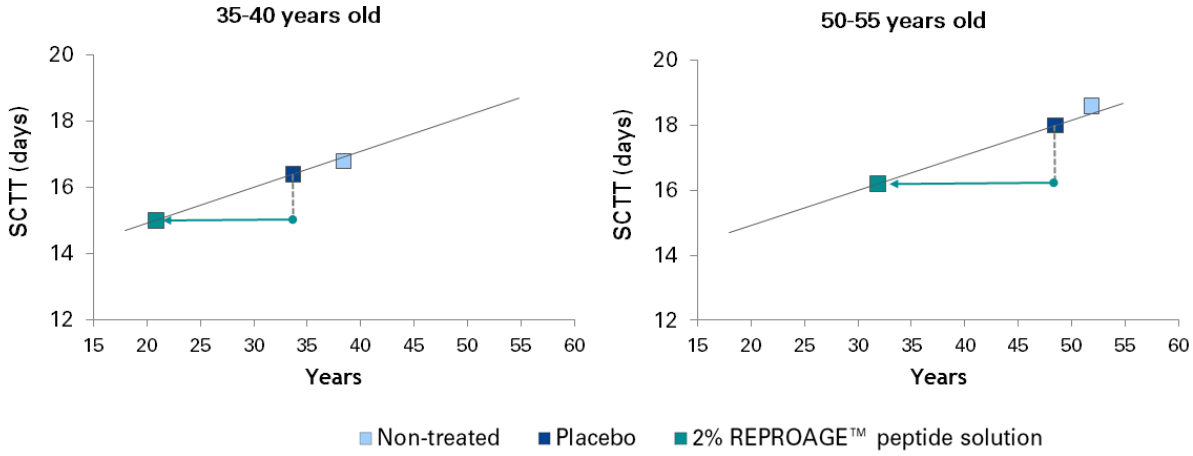


Fig.10. Correlation between volunteers' age and SCTT and the interpolated years of rejuvenation.

With respect to placebo, the SCTT in the 35-40 years old subjects treated with the active cream corresponded to that **of 13 years younger skin**.

Skin renovation equivalent to 17 years younger skin (in 50-55 years old volunteers).

Skin roughness

The properties of the skin surface were analyzed after twice a day application of 2% REPROAGE™ *peptide solution* and a placebo cream to half face for 56 days.

Skin roughness in the area underneath the eyes was measured by means of a microtopography imaging system and the average surface roughness (Sa) was obtained.

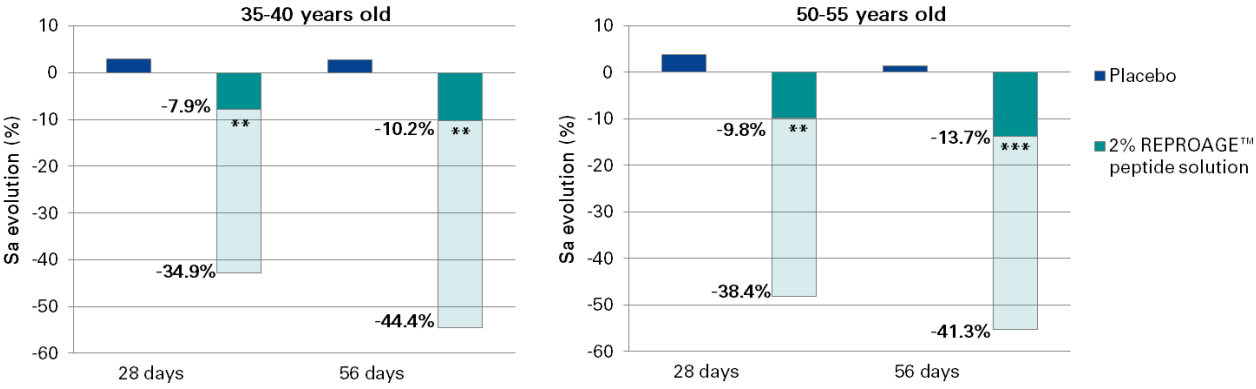


Fig.11. Changes in skin roughness under different conditions. Average and maximal values of Sa reduction are shown. (vs initial time: **p<0.01; ***p<0.001; vs placebo: *p<0.05 in 35-40 years old; **p<0.01 in 50-55 years old).

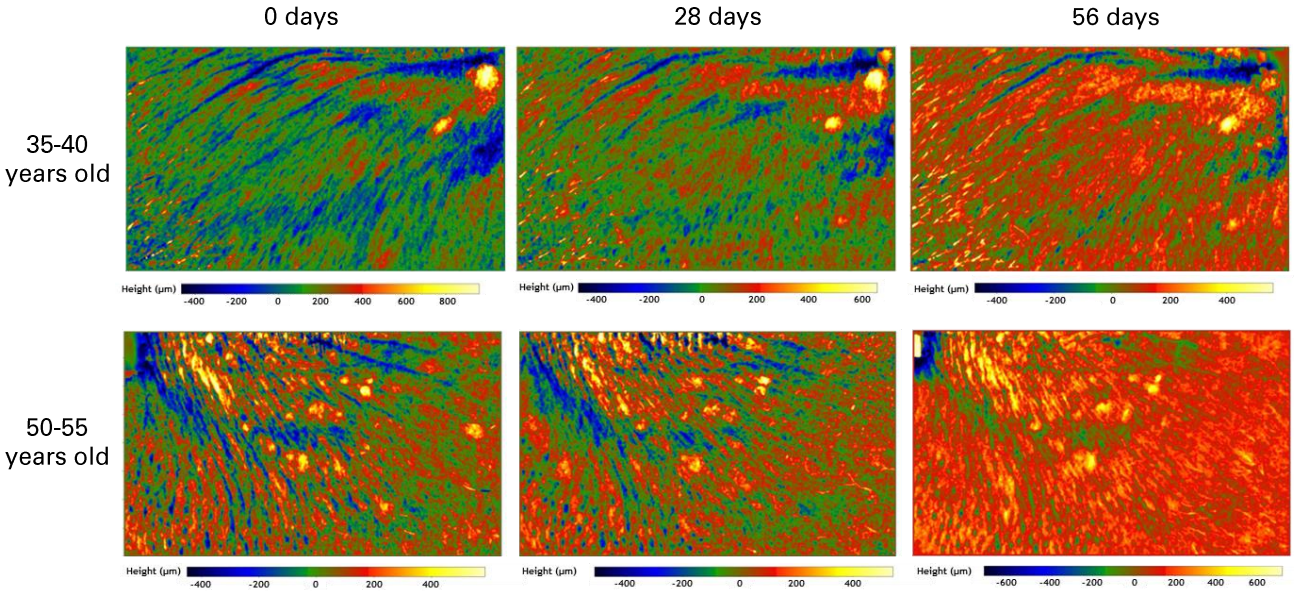


Fig.12. Reconstructed 3D images of skin roughness. The reduction in surface depth can be seen as a tendency to change from blue to red colors.

Irregularities in the skin surface **decreased** after the active treatment, with significant results **compared to placebo**.

Up to 41.3% decrease in roughness in 50-55 years old volunteers.

RADIANT AND BETTER-LOOKING SKIN

To determine changes in the skin complexion, an *in vivo* test was carried out

20 female volunteers (35-55 years old) applied a cream containing 2% REPROAGE™ *peptide solution* to the face twice a day.

After 56 days of treatment, skin luminance (L*) was measured from high resolution photographs by means of the appropriate image analysis software.

Fig.13. Photographs of one volunteer before and after 8 weeks of treatment.

Skin luminance increased by 1.5% (*p<0.05) after 56 days and the skin of the volunteers showed a **general improvement** in appearance.

Overall improvement in skin radiance and complexion.

Ingredient properties

REPROAGE™ *peptide*:

- is an hexapeptide **inspired by epigenetics** to help the self-renewing properties of the epidermis last longer.
- **reduces** the levels of **miR-145**, which is involved in the regulation of epidermal differentiation.
- **enhances** the expression of SOX2, OCT4 and KLF4, the **stem cell transcription factors**, targets of miR-145, suggesting an increase in stemness.
- may induce **reprogramming of epidermal cells**, as it modulated the profile of expression of keratins typical of the basal and suprabasal layers, favoring the recovery of the basal state.
- boosted p63 in the basal epidermis by 110.9%, suggesting a greater **regenerative potential**.
- applied *in vivo* as a 2% solution, aided in accelerating skin regeneration, rejuvenating the **self-renewal** rate of the epidermis by **13 and 17 years** for the younger and older subjects respectively.
- might help obtain a **smoothening** effect, as skin roughness was reduced by 13.7% on average in the older volunteers after 56 days and up to 44.4% in the younger volunteers.
- when applied to facial skin, was associated with an increase in **luminance** of 1.5% and the observation of a better appearance of the complexion of the volunteers.

Cosmetic applications

REPROAGE™ *peptide* can be incorporated into any anti-aging formulation seeking to cope with the slowdown in skin regeneration that takes place with age.

By aiding the natural process of self-renewal in the skin, it can provide formulations the capability to reveal a revitalized complexion. It can be used in products designed to rejuvenate the skin and improve its appearance, enhancing radiance and surface smoothness.

Technical data

INCI NAME OF THE ACTIVE INGREDIENT

Active ingredient	INCI name
REPROAGE™ *peptide*	Acetyl Hexapeptide-8

PRESENTATION AND PRESERVATIVE

Translucent solution containing 0.05% Acetyl Hexapeptide-8.

Code	Product presentation	Preservative
PD280	REPROAGE™ *peptide solution*	-

Application data

PROCESSING

REPROAGE™ *peptide* can be formulated in the aqueous phase of formulations, in the final step of the manufacturing process. In case of preparing an emulsion, it is recommended to add it once the emulsion is formed and at a temperature below 40 ºC.

Recommended pH range between 5.0 and 7.0 for REPROAGE™ *peptide*.

INCOMPATIBILITIES

Not expected.

SOLUBILITY

Soluble in water and glycols. Insoluble in oils and silicones.

DOSAGE

A dosage of 2% of REPROAGE™ *peptide solution* is recommended in final cosmetic formulations.

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