

Royalactin-like peptide to regain skin vitality

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Imagine – you have just been born and your future looks bleak. You have been cared for only the first days after birth and then you are already forced to work: cleaning the home that you share with your many siblings, collecting food not for yourself but for the community, keeping watch and defending your home with your life, if necessary. You will not have a single minute of free time and never go on vacation. You will age fast and within a few weeks you have worked yourself to death. And the worst part – you will never have known true love. How different is the life of your sister. She will be pampered, she never has to leave home, she will be surrounded by loyal servants, who feed her, clean her and protect her. She exceeds you in weight and size, she has many lovers and she will be mother to thousands of offspring. Your sister is more resistant to environmental stress factors, ages much more slowly and will live 10 times longer than you. She has nothing to fear – because she is the queen and you are just a common worker bee.

One genome – two fates

The honeybee (*Apis mellifera*) forms two female castes: the queen and the worker. While the queen bee is large in size and specialised in reproduction, the workers are small and engaged in activities for maintaining the colony. The queen bee lives far longer than any other bee in the hive. She is more resilient with respect to extrinsic environmental stress factors, such as thermal stress and has a slower intrinsic ageing process in comparison to worker bees.¹ Despite their vastly different appearance, physiology and behaviour, queen and worker bees share the same genome. They differ only in which genes are activated and hence which proteins are produced to serve specific functions. Indeed, young queen larvae produce higher levels of proteins involved in amino acid metabolism, energy production and repair, a prerequisite for tissue growth and maintained homeostasis. How does it

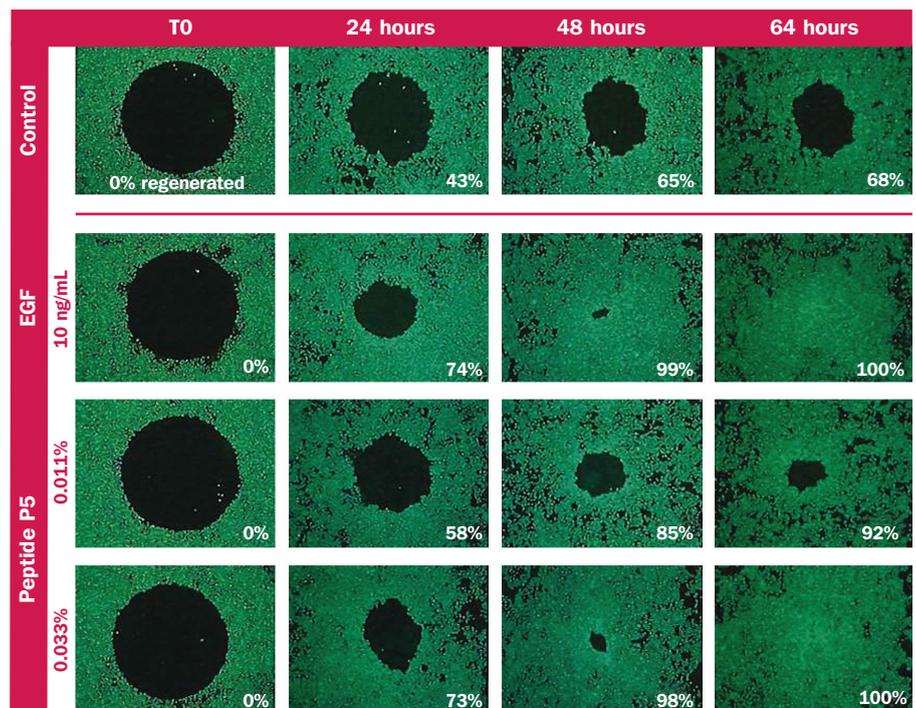


Figure 1a: Wound healing assay. The peptide stimulates NHEK proliferation/migration. The effect is similar to the reference EGF.

work? Honeybees modify their genetic code by consuming a special diet called royal jelly (RJ). This peculiar juice leaves chemical 'markers' on the DNA, which modify gene expression without actually

changing the genetic code – a process referred to as 'epigenetic' modification ('on top of the genome'). Thus, honeybees have a clever way of generating two contrasting organisms by using environmental factors to influence the same genome. RJ is the epigenetic trigger that determines a honeybee larvae's fate.



Queen bee surrounded by workers.

Royalactin: the 'queenmaker' in Royal Jelly

The mechanism of action and active ingredient of RJ was a mystery until 2011, when a Japanese scientist discovered the 'queenmaker' protein royalactin. This 57 kDa protein is the one crucial factor that drives all the changes needed to make a queen bee.² Interestingly, the effects of royalactin are not just limited to bees. Scientific studies have revealed a kind of 'cell doping' effect in other insects. For example, royalactin increases longevity in fruit flies. Likewise, feeding the nematode worm

C. elegans with royalactin delays ageing and increases its lifespan.³ In mammals, on the other hand, royalactin was reported to enhance proliferation in rat liver cells.⁴ All of these effects are due to the putative interaction of royalactin with the epidermal growth factor (EGF) receptor.² In other words, activation of the EGF-signalling pathway not only triggers the development of a regular honey bee larva into a queen, but it also promotes tissue regeneration and delays senescence in human skin cells.^{5,6}

Development of the royalactin-like peptide

Interestingly, only fresh RJ is fully potent. This is due to the quick degradation of royalactin after a short time of storage. Therefore, the isolated royalactin cannot be used as a test compound in cell culture studies. Our R&D team met the challenge by developing the pentapeptide-TRSEL (peptide P5), which is intended to mimic an active site of royalactin. This peptide is based on the highly conserved Arginine-domain found in natural ligands of the EGF-receptor, such as the epidermal growth factor and the transforming growth factor alpha. The Arg-domain is recognised by the EGF receptor and responsible for its activation. Encapsulation of the peptide into a nano-structured lipid matrix ensures its delivery to the target skin layer without losing any activity. The peptide is commercialised by Mibelle Biochemistry under the trade name RoyalEpigen P5 (INCI: Pentapeptide-48, Hydrogenated Lecithin, Glycerin, Butyrospermum parkii (shea) Butter, Phenethyl Alcohol, Ethylhexylglycerin, Maltodextrin, Water).

Methods

Proteomics vs. genetics

Proteomics is the study of all the proteins expressed in a cell. Proteins are the functional molecules in the cells and represent actual conditions. Genomic studies may not fully reveal actual conditions in the cell due to regulation at the RNA and protein level that are not captured.

Proteomic analysis

Primary keratinocytes from a single adult donor were either cultured in a) standard medium; b) specific senescence-inducing medium; or c) senescent-inducing medium supplemented with 1% of the pure peptide P5 (not encapsulated). At the end of the ageing process cells were lysed and subjected to proteomic analysis (MRM proteomic technology) for the detection and quantification of a predefined set of proteins.

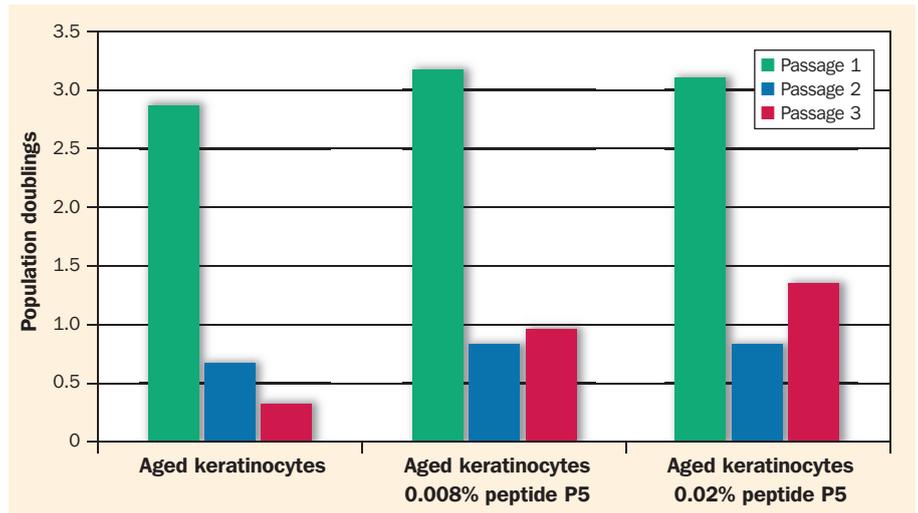


Figure 1b: Peptide P5 enhances proliferation and longevity in aged keratinocytes.

Wound healing assay 2D cell model

Normal human epidermal keratinocytes (NHEK) were grown and differentiated with a particular culture medium. An artificial wound was made in the confluent cell monolayer by using a cell seeding stopper. Once adhesion was completed, the stopper was removed. Cells were labelled with calcein-AM. Cells were then allowed to proliferate and migrate back into the artificial wound in the presence of peptide P5 (encapsulated) at 0.033% and 0.01% or the reference compound (EGF at 10 ng/mL). The wound recovery rate was monitored by using a Nikon Diaphot 300 microscope. Images were taken using a Nikon DX-1200 camera. Experiments were performed in triplicate for verification.

added at 0.008% and 0.02% concentration. An untreated culture served as control. The number of population doublings was determined for cells grown for one, two and three passages.

Gene expression

NHEK were cultured in culture medium containing the peptide P5 (encapsulated) at 0.03% and 0.1% concentration. An untreated culture served as control. After 24 hours of incubation, cells were harvested and subjected to gene expression analysis by using quantitative PCR (LightCycler® system; Roche Molecular System Inc.). This experiment was performed in triplicate for verification.

In vivo studies

A cream containing 2% peptide P5 (encapsulated) was tested in a clinical study with 20 healthy volunteers, average age 49. The cream was applied twice daily for 28 days to the inner side of the

Keratinocyte proliferation assay

Primary human keratinocytes were cultivated in a low-energy medium, resembling the conditions in aged skin (CellnTec). Peptide P5 (encapsulated) was

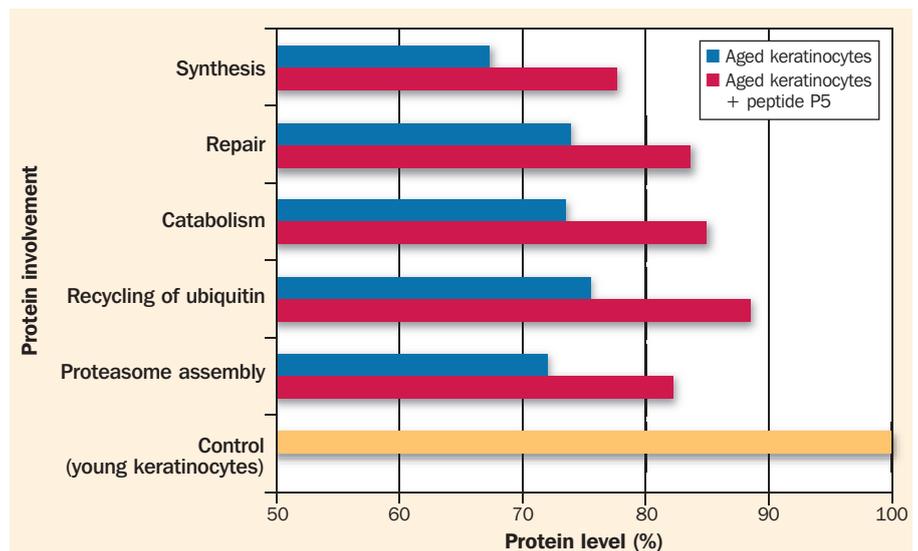


Figure 2: Effect of peptide P5 on protein turnover in aged keratinocytes. Young keratinocytes serve as control (100%).

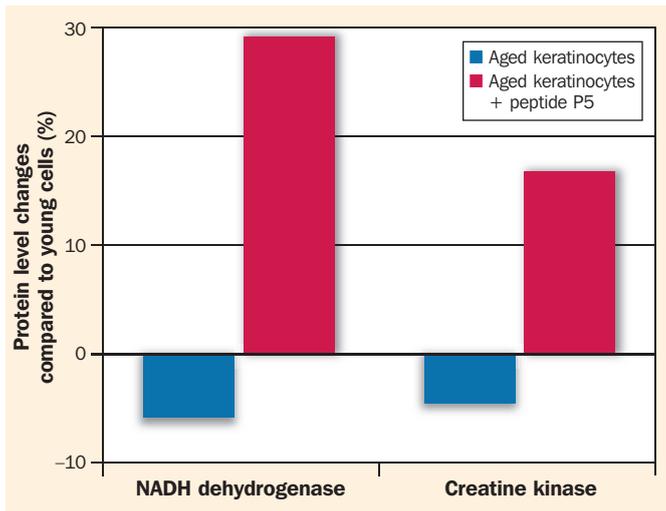


Figure 3: Effect of peptide P5 on the expression of important mitochondrial components.

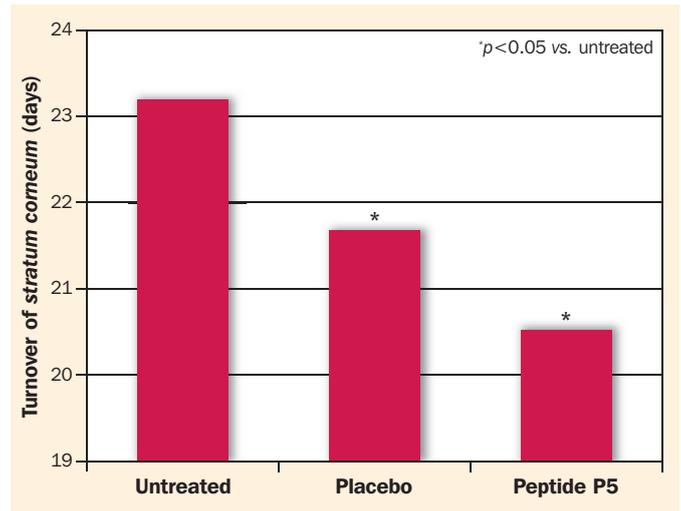


Figure 4: Improvement of skin renewal capacity.

forearm and the face. The other forearm was treated with a placebo cream. 8-12 hours after the last product application on days 14 and 28, skin firmness and smoothness on the forearms and skin evenness on the face were measured by means of the Cutometer MPA 580, PRIMOS® and Minolta Chromameter CR 400. Cell renewal time was determined on the inner side of the forearms by using the dansyl chloride technique.

Results: *in vitro* studies

Peptide P5 triggers skin cell's regenerative capacity

The regenerative potential of a cosmetic active can be tested in an *in vitro* wound healing assay. In short, the basic step involves creating an 'artificial wound' in a cell monolayer, capturing images of the preparation at the beginning and at regular intervals during cell migration, which eventually closes the wound. Peptide P5

shows the remarkable capacity to accelerate proliferation and migration of keratinocytes to efficiently close the lesion (Fig. 1a). The obtained images indicate a potent regenerative effect of the peptide, indeed very similar to the reference compound EGF.

These results were supported by a proliferation study on aged keratinocytes. The proliferation rate of *in vitro* aged keratinocytes declines continuously until it

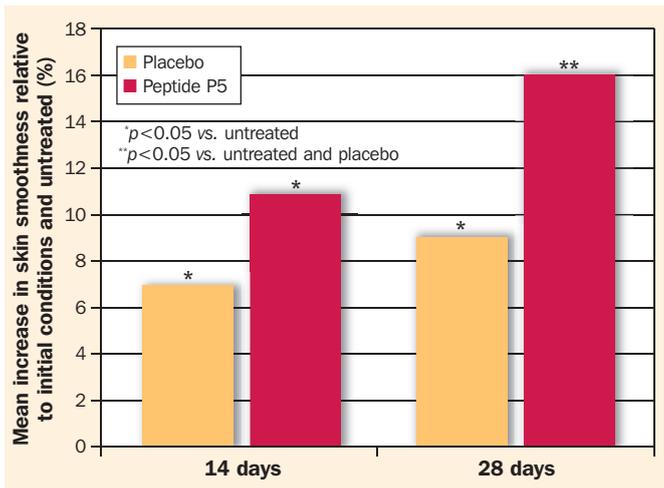


Figure 5: Smoother skin texture after four weeks of treatment with a cream containing 2% peptide P5.

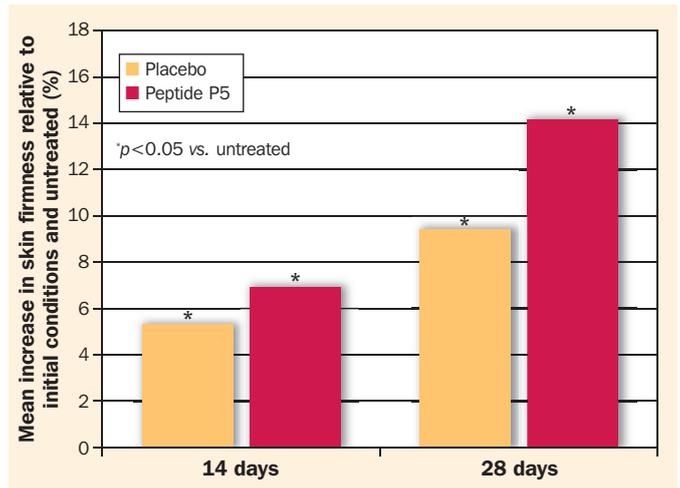


Figure 6: Improvement of skin firmness after four weeks of treatment with a cream containing 2% peptide P5.

ceases completely. Addition of peptide P5 to the cell cultures resulted in a three- to fourfold increase in population doublings compared to the untreated control (Fig. 1b).

Peptide P5 triggers Skp-1 expression and improves overall protein quality

By activating EGF signalling, royalactin clearly targets a crucial regulator of the ageing process. Scientific studies have shown that activation of the EGF pathway leads to the stimulation of the Skp-1 like adaptor, a key-limiting factor in the ubiquitin-proteasome system (UPS).⁷ The role of UPS is to recycle damaged proteins and to maintain high protein quality. A highly active UPS was found in skin cells from centenarians. A gene expression study performed on human keratinocytes revealed that the peptide P5 stimulates the synthesis of Skp-1 in keratinocytes in a concentration-dependent manner. 0.1% peptide added to the cell culture resulted in a significant upregulation of Skp-1 by 197% (control 100%). Furthermore, a proteomic study performed on senescent keratinocytes confirmed that the peptide attenuates the age-dependent reduction of proteasome subunits, ribosomal components and chaperones, thus promoting protein turnover and protein repair (Fig. 2).

Peptide P5 supports mitochondrial stability and ensures energy production for cellular processes

Cellular processes like the UPS depend on energy in form of adenosine triphosphate (ATP), which is produced in small organelles called mitochondria. The loss of mitochondrial function is one of the main hallmarks of ageing. Data collected from our proteomic study indicate that some mitochondrial components start to decline in aged keratinocytes. Among those are:

The creatine kinases, which serve as reservoir and energy transport system. A reduced level of creatine kinases has been linked to a reduced production of dermal matrix components like collagen.⁸ Another example is the NADH dehydrogenase 1, a key component of the energy transport chain; its decrease was shown to augment skin pigmentation in elderly people resulting in uneven skin tone.⁹ Peptide P5 substantially increases the level of these enzymes in aged skin cells, as seen in Figure 3.

Clinical study Enhanced skin quality

Restoring a healthy population of various proteins in aged keratinocytes correlates with visible skin benefits as demonstrated in our *in vivo* studies. Twenty volunteers with inhomogeneous skin tone participated in a placebo-controlled study. A cream containing 2% of peptide P5 was applied to the inner side of the forearm and the face. The renewal time of the *stratum corneum* decreased by almost 3 days (Fig. 4) resulting in a much smoother skin surface (Fig. 5). An effect was observed in 100% of the volunteers. Moreover, the peptide was found to significantly improve skin tone homogeneity in 80% of the volunteers. Furthermore, peptide P5 preserved the biomechanical properties of the skin: 95% of the volunteers showed an improved skin firmness (Fig. 6).

Conclusion

The outside world can have a direct effect on cellular DNA. Peptide P5, based on the queen maker protein royalactin, consists of a 5 amino acid sequence that is prominent in several growth factors. The peptide promotes Skp-1 upregulation in aged skin cells resulting in rejuvenation of specific protein populations. Stimulating the production of important mitochondrial

components helps to preserve the skin's natural vitality. Our new active ingredient keeps the skin supple and gives it a smoother texture and more even appearance.



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