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The regulatory role of the tetrapeptide AcSDKP in skin and hair physiology and the prevention of ageing effects in these tissues – a potential cosmetic role

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Synopsis

The naturally occurring tetrapeptide acetyl-N-Ser-Asp-Lys-Pro (AcSDKP) recognized as a potent angiogenic factor was shown recently to contribute to the repair of cutaneous injuries. In the current article, we report the ability of AcSDKP to exert a beneficial effect on normal healthy skin and scalp and to compensate for the ageing process. *In vitro* AcSDKP at 10^{-11}–10^{-7} M significantly stimulates the growth of human keratinocytes, fibroblasts and follicle dermal papilla cells. Moreover, it enhances the growth of human epidermal keratinocyte progenitor and stem cells as shown in a clonogenic survival assay. Topical treatment of *ex vivo* cultured skin explants with 10^{-5} M AcSDKP increases the thickness of the epidermis and upregulates the synthesis of keratins 14 and 19, fibronectin, collagen III and IV as well as the glycoaminoglycans (GAGs). In the *ex vivo*-cultured hair follicles, AcSDKP promotes hair shaft elongation and induces morphological and molecular modifications matching the criteria of hair growth. Furthermore, AcSDKP at 10^{-11}–10^{-7} M was shown to improve epidermal barrier, stimulating expression of three protein components of tight junctions (claudin-1, occludin, ZO-1) playing an important role in connecting neighbouring cells. This tetrapeptide exercises also activation of SIRT1 implicated in the control of cell longevity. Indeed, a two-fold increase in the synthesis of SIRT1 by cultured keratinocytes was observed in the presence of 10^{-11}–10^{-7} M AcSDKP. In conclusion, these findings provide convincing evidence of the regulatory role of AcSDKP in skin and hair physiology and suggest a cosmetic use of this natural tetrapeptide to prevent skin ageing and hair loss and to promote the cutaneous regeneration and hair growth.

Résumé

Le tétrapeptide acétyl-N-Ser-Asp-Lys-Pro (AcSDKP) est connu comme un stimulateur de l'angiogenèse et joue un rôle bénéfique dans la réparation des lésions cutanées. Les résultats de cette étude ont permis de mettre en évidence des nouvelles propriétés biologiques de ce peptide vis-à-vis de la peau et du cuir chevelu. En effet, AcSDKP stimule significativement la croissance des kératinocytes, des fibroblastes et des cellules de la papille dermique du follicule pileux en culture ainsi que des cellules souches et des cellules progénitrices des kératinocytes de l'épiderme humain. Nous avons montré que l'application topique d'AcSDKP sur des explants de peau humaine maintenus en survie stimule significativement la production des collagènes III et IV, de la fibronectine et des glycosaminoglycanes (GAGs) ainsi que la synthèse des kératines 14 et 19. D'autre part, le traitement avec AcSDKP des explants de cuir chevelu ainsi que des cheveux isolés maintenus en survie favorise l'allongement de la tige du cheveu et induit des modifications morphologiques et moléculaires associées à la croissance des cheveux. De plus, AcSDKP améliore la qualité de la barrière épidermique, en stimulant *in vitro* l'expression des trois composantes protéiques des jonctions serrées (claudine-1, occludine, ZO-1) qui jouent un rôle important dans la connexion des cellules adjacentes. Ce tétrapeptide stimule également l'expression de la protéine SIRT1 qui est impliquée dans le contrôle de la longévité cellulaire. En effet, on observe une augmentation de 200% de la synthèse de SIRT1 par les kératinocytes en culture en présence d'AcSDKP. Ces résultats mettent en évidence le rôle régulateur d'AcSDKP dans la physiologie de la peau et des cheveux et suggèrent une utilisation cosmétique de ce tétrapeptide pour prévenir le vieillissement cutané et la chute des cheveux.

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Introduction

Skin and hair ageing is a complex multi-dimensional biological process that is a consequence of both intrinsic chronological ageing that occurs with time, and extrinsic ageing caused by environmental factors [1]. The dramatic increase in the ageing population and the psychosocial impact of skin and scalp changes with age has created a demand for effective interventions. The considerable progress made during the last years in the understanding of the cellular, biochemical and molecular changes associated with ageing allows the development of different approaches to reduce and to repair the undesirable effects of programmed ageing and environmental injury [2].

Skin changes are one of the most visible signs of ageing. Symptoms of chronological ageing are among other's dry and thin skin as well as fine wrinkles because of the diminished production of

collagen [1, 3]. On the other hand, the external insults, including most of all ultraviolet sunlight, induce damages to the collagen and elastic fibres in the skin, which also becomes loose and wrinkled [1, 3, 4]. Photoageing is thus a key factor of elasticity and tightness process deterioration. The accumulating scientific evidence reveals that chronological ageing and photoageing share fundamental molecular pathways [5]. This convergence of the molecular basis of two ways of skin deterioration provides new opportunities for the development of new anti-ageing therapies.

The scalp and hair are also subject to both chronological ageing and external insults such as solar irradiation or air pollution [6, 7]. Scalp deterioration comprising hair shaft and hair follicle ageing results in hair thinning and a decrease in hair production (alopecia).

Many products claim to revitalize ageing skin or reduce wrinkles, but only a few have been really approved for their efficacy. Significant progress was also made in elaboration/discovering/identification of factors claiming to be useful for the treatment and prevention of hair ageing. However, the new drugs, which can target overall and effectively hair growth, are still awaited. Recently, peptides have been introduced into skin/haircare product formulations achieving the desired effect [8–10].

In the current report we focus on the natural tetrapeptide acetyl-N-Ser-Asp-Lys-Pro (AcSDKP) whose beneficial effects on skin injuries were previously demonstrated [11]. Locally administrated AcSDKP rescued the impaired vascularization of ischaemic experimental skin flaps and increased their survival. Moreover, an *ex vivo* assay using human skin explants exhibited accelerated healing of skin lesions induced by UVB irradiation following topical AcSDKP treatment. Because the ageing and wound-healing processes are tightly linked sharing common molecular targets, we have undertaken these studies on the anti-ageing potential of AcSDKP.

AcSDKP is present in the blood at nanomolar concentrations and is formed *in vivo* by the enzymatic processing of the N-terminus of thymosin β4 [12]. This tetrapeptide was reported as a potent physiological regulator of angiogenesis [13, 14]. Indeed, it interacts directly with endothelial cells and elicits an angiogenic response *in vitro* and *in vivo*. Neovascularization following *in vivo* AcSDKP administration markedly reduces the extent of ischaemic damage in experimentally induced cutaneous injury, myocardial infarction and hindlimb ischaemia [11, 14, 15].

Thus, the well-defined angiogenic properties of AcSDKP allow one to expect an improvement of blood supply when it is applied to the skin or scalp. Indeed, in general the process of angiogenesis, during which new blood vessels are formed, is impaired in the aged [16]. Therefore, with ageing, cutaneous blood vessels undergo pronounced alterations and a reduction in the cutaneous microvasculature in the skin of elderly individuals is well documented [17]. Moreover, it has long been noted that hair follicle cycling is associated with substantial angiogenesis [18]. Taking into account the potential of AcSDKP to improve health and beauty/appearance of skin and hairs, we have investigated in further detail the effect of this peptide on the molecular parameters related to ageing process.

The present studies have shed light on the potential of AcSDKP to stimulate epidermal and dermal metabolism and thus to fight against natural deterioration of skin. All the findings reported in this article strongly suggest that the application of this natural peptide in skin care would correct not only existing signs of ageing but would also slow chronological ageing. Moreover, the potent effect of AcSDKP on a number of molecular targets related to hair physiology suggests its cosmetic use to prevent scalp ageing and to stimulate hair growth and/or slow hair loss.

Materials and methods

Peptide

Sterile samples of synthetic AcSDKP (MW 487, purity > 95% as determined by reverse phase high-performance liquid chromatography) were provided by Genepep (Saint-Jean de Védas, France).

Cell culture

Human keratinocyte cell line (HaCaT, CLS, Eppelheim, Germany) were grown in Dulbecco' modified Eagle medium (DMEM) containing 4.5 g L^{-1} glucose supplemented with 10% foetal calf serum (FCS) and 1% glutamine (Lonza Sales LTD, Basel, Switzerland). Primary normal human dermal fibroblasts (NHDF-Ad) derived from adult skin tissue were purchased from Lonza and cultured according to the supplier's instructions in fibroblast growth medium containing human basic fibroblast growth factor (bFGF), insulin and 2% FCS. Human follicle dermal papilla cells (HFDPC) isolated from human dermis originating from lateral scalp were purchased from Tebu-Bio (Le Perray en Yvelines, France) and grown in Follicle Dermal Papilla Cells Medium containing 4% FCS, 0.4% bovine pituitary extract, 1 ng mL^{-1} bFGF and 5 μg mL^{-1} insulin (Tebu-Bio). The cells were maintained at 37°C in a humidified atmosphere containing 5% CO_2.

Cell growth assay

Cell number was estimated using CellTiter-Blue™ assay (Promega GmbH, Mannheim, Germany) according to the manufacturer's instructions. Cells were seeded in 96-well plates (5×10^3 cells per well) containing 50 μL growth medium. After 24 h of culture, the medium was removed and replaced by DMEM supplemented with 0.5% FCS and then a day later, the cells were supplemented with 50 μL of AcSDKP (10^{-11}, 10^{-9}, 10^{-7} M final concentration) dissolved in DMEM supplemented with 0.5% FCS. After 72 h of incubation, 20 μL of resazurin was added for 2 h before recording fluorescence (λ_{ex} = 560 nm, λ_{em} = 590 nm) using a Victor microtitre plate fluorimeter (PerkineElmer, Waltham, MA, U.S.A.). Experiments were performed in triplicate and the cell proliferation rate is represented as percentage of the control value (vehicle-treated cells).

Colony-forming efficiency assay

Clonogenic assay or colony formation assay is an *in vitro* cell survival assay based on the ability of a single cell to grow into a colony.

Human keratinocyte cells

Experiments of clonogenic survival assays have been performed using human interfollicular epidermal keratinocytes of neonatal foreskin samples from routine circumcisions and obtained after informed consent of the patient's parents. The ethical and security aspects concerning the use of human cells and tissues are in accordance with the CODECO number DC-2008-228, reviewed by the ethical research committee IDF-3.

Human neonatal biopsies were incubated overnight at 4°C in a solution containing DMEM, dispase 2.4 U mL^{-1}, trypsin 0.5 g L^{-1} and 2.5% antibiotics from Life Technologies (Saint-Aubin, France). Epidermal sheets were then separated from the dermis. The epidermis was incubated in trypsin-EDTA for 20 min at 37°C. Cell

dissociation was performed by gentle pipetting and filtering on 70 μm cell strainers (BD Biosciences, Le Pont-de-Claix, France) to obtain a single-cell suspension. Then, keratinocytes were centrifuged at 200 *g* for 15 min and cell viability was assessed using trypan blue (Sigma, Saint-Quentin Fallavier, France).

Cell staining and sorting

Basal keratinocyte subpopulations were freshly isolated from tissue samples on the basis of two cell-surface markers, an adhesion molecule (integrin-α6) combined with a proliferation-associated marker (transferrin receptor, Trf-R) according to Kaur's laboratory protocol [19]. Cell immunostaining was performed in PBS-2% bovine serum albumin (Sigma) and required an incubation with mouse γ-globulins (Beckman Coulter, Villepinte, France), followed by staining with R-phycoerythrin-conjugated rat anti-human CD49f/integrin-α6 (Itg-α6) antibody (clone GoH3; BD Biosciences) and streptavidin allophycocyanin mouse anti-human CD71/transferrin receptor (Trf-R) antibody (BD Biosciences).

Then, cell populations were sorted by flow cytometry (MoFlo; Dako, Trappes, France). Trf-R expression does successfully separate the integrin-α6 bright (Itg-α_6^{bri}) proliferative compartment of human epidermis into quiescent [keratinocyte stem cells (KSC)] [int-α_6^{bri} CD71^{dim}] and cycling [transient amplifying cells (TA)] [int-α_6^{bri} CD71^{bri}] tissue reconstituting cells [19–21].

Clonogenic survival assay

Human primary sorted basal keratinocytes (KSC and TA) were seeded at a clonal density of 5 keratinocytes cm^{-2} onto lethally irradiated human fibroblasts in Green L1 medium. This culture medium contained DMEM and Ham's F12 media (3 : 1 mixture; Life Technologies), 10% FCS (Thermo Fisher Scientific, Villebon-sur-Yvette, France), 10 ng mL^{-1} epidermal growth factor (Millipore SAS, Molsheim, France), 5 μg mL^{-1} transferrin, 5 μg mL^{-1} insulin, 0.4 μg mL^{-1} hydrocortisone, 180 μM adenine, 2 mM tri-iodothyronine (Sigma), 2 mM L-glutamine (Life Technologies), and 100 U mL^{-1} penicillin/streptomycin (Life Technologies). Medium was renewed every 2 days.

Cells were allowed to attach to culture dishes overnight at 37°C in a humidified atmosphere with 5% CO_2. After 24 h, 100 μL of AcSDKP solution was added to the culture medium at two final concentrations (10^{-5} M and 10^{-10} M). Cells were continuously exposed to AcSDKP and the size of individual colonies was quantified after 2 weeks of culture. Colony staining was performed with eosin and blue reagent (RAL 555) from RAL Diagnostic (Martillac, France). Keratinocyte colony-forming efficiency (CFE) corresponds to the ratio of the number of formed colonies to the number of seeded cells. The CFE values were determined for estimating the proliferation of keratinocytes treated with AcSDKP at each concentration and compared with the control vehicle-treated cells. Three experiments were carried out using independent foreskin samples and statistical comparisons were performed using the Student's *t*-test. Data are presented as mean ± SEM values and $P < 0.05$ was considered statistically significant.

Ex vivo human skin-explant assay

The human skin specimens were obtained after informed consent from healthy patients undergoing corrective plastic surgery. Time laps between surgical excision of the skin and its use never exceeded 4–6 h. Subcutaneous fat tissue was removed and skin tissue was washed, cut into round 1 cm^2 sections and transferred immediately in six-well plates. The explants were placed epidermal surface upwards on a stainless steel grill allowing the immersion of their lower surface in skin survival culture medium (Bio-Ec Laboratory, Longjumeau, France) supplemented with gentamycin (50 μg mL^{-1}) and amphotericin B (205 μg mL^{-1}). All skin specimens were cultured for 8 days at 37°C in a humidified atmosphere containing 5% CO_2. Half of the medium was replaced every 2 days. AcSDKP solution in saline (10^{-5} M and 10^{-8} M) was applied to the explant's dry upper surface every 2 days for 1 week. The controls were treated with saline. At day 0, 2, 4, 6 and 8, three explants of each batch were harvested for histological analysis. Each skin explant was divided into two parts, one part was fixed in standard Bouin solution for 48 h, dehydrated and embedded in paraffin, the second one was frozen in liquid nitrogen and stored at −80°C for later processing.

Histology and immunohistochemistry

To visualize general skin morphology, 5 μm sections of paraffin-embedded tissues were deparaffinized, stained using Masson's trichrome (Labonord S.A., Templemars, France) and analysed by light microscopy. Cryostat sections (7 μm thick) of frozen skin explants were fixed in acetone and then analysed for the expression of skin macromolecular components using immunochemistry. The antibodies used in the study included mouse monoclonal anti-K14 (clone RCK 107; Tebu-Bio), mouse anti-K19 (clone b 170; Menarini Diagnostics, Rungis, France), mouse anti-fibronectin (clone TV-1; Euromedex, Mundolsheim, France), rabbit polyclonal anti-collagen III (Tebu-Bio) and goat polyclonal anti-collagen IV (CliniSciences, Nanterre, France). Glycosaminoglycans (GAGs) were stained according to combined alcian blue-PAS method [22]. The biotinylated goat anti-mouse and goat anti-rabbit secondary antibodies (Amersham Biosciences, Saclay, France) were revealed either with streptavidin-FITC or streptavidin-alkaline phosphatase from Tebu-Bio. Nuclei were labelled using propidium iodide (Sigma). Images were taken on a DLMB microscope (Leica Microsystems SAS, Rueil-Malmaison, France).

Ex vivo culture of human hair follicle

This study was performed using the explants of human hair follicles obtained from the donors scalp. Hair follicles including hair shafts were isolated by microdissection, placed individually in 48-well plates and maintained in survival for 15 days in conventional cell culture conditions (37°C, 5% CO_2) in William's medium. At day 0, AcSDKP was added to the culture medium at three concentrations: 10^{-4}, 10^{-7} and 10^{-10} M. The culture media containing the tested compound were renewed every 2 days.

The individual hair follicles were photographed at d0, d3, d6, d8, d10, d13 and d15 of treatment, using a microscope and a CCD camera coupled to a software acquisition and archiving. The length (μm) of each hair was estimated using the measuring module LEICA IM1000 measures. At d8 and d15 of treatment, six hair from each batch were collected and prepared for histological studies. Three explants from each batch were fixed in regular Bouin solution and three others were frozen and stored at −80°C. The follicle morphology was visualized following staining with Masson's trichrome. The mouse anti-laminin 5 (clone P3E4; Santa Cruz Biotechnology, Heidelberg, Germany), goat polyclonal anti-collagen IV (CliniSciences) and mouse anti-K19 (Menarini Diagnostics) antibodies were used for immunohistochemical studies. The mitotic index of follicular structures was determined following the immunostaining with

anti-Ki67 antibody (clone 7B11; CliniSciences) that labels proliferating cells [23].

Immunocytochemistry

HaCaT keratinocytes or NHDF fibroblast cells cultured on a Lab-Tek chamber slide (VWR International, Strasbourg, France) were treated with AcSDKP at different concentrations for 24 h. The cells were then washed once with PBS prior to fixation with 4% paraformaldehyde for 20 min. The fixed cells were washed three times in PBS and next permeabilized with 0.1% Triton X-100 (Sigma) in PBS for 3 min at room temperature. The cells were washed again three times in PBS and preincubated for 2 h in PBS containing 3% bovin serum albumin (BSA) and 10% goat serum to block non-specific binding sites. Cells were stained with the primary antibody diluted in PBS with 3% BSA overnight at 4°C. The antibodies used in the study included rabbit polyclonal anti-collagen I, rabbit anti-SIRT1 (Santa Cruz Biotechnology), rabbit anti-claudin-1, rabbit anti-occludin and rabbit anti-ZO-1 (Zymed Laboratories, San Francisco, CA, U.S.A.). The labelled cells were washed three times in PBS containing 1% BSA and incubated for 2 h at room temperature with Alexa Fluor 488-labelled goat anti-rabbit antibody (Life Technologies). Alexa Fluor 546 labelled-phalloidin which binds selectively to F-actin was used for actin staining and Hoechst 33342 for nuclear staining (Life Technologies). At the end of incubation, the cells were washed once in PBS and mounted in fluorescent mounting medium (Dako). Images were acquired using a Nikon TE2000E fluorescent microscope (Nikon, Champigny-sur-Marne, France) equipped with a Nikon DXM1200F digital camera.

Western blot analysis

Human keratinocytes (HaCaT) or fibroblasts (NHDF) were seeded in six-well plates at a density of 2×10^5 cells mL^{-1} with complete culture medium. The cultured control and AcSDKP-treated cells were rinsed with cold PBS and protein were extracted with cell lysis buffer (Cell Signaling Technology, Danvers, MA, U.S.A.) supplemented with protease inhibitors (Sigma). Protein (20 μg) were resolved in 10% SDS-PAGE and transferred onto nitrocellulose membrane (Millipore). After blocking with 5% w/v non-fat dry milk in TBS, membranes were incubated overnight at 4°C with the following primary antibodies: rabbit anti-SIRT1 (Santa Cruz Biotechnology), rabbit anti-claudin-1, rabbit anti-occludin and rabbit anti-ZO-1 (Zymed Laboratories) followed by goat anti-rabbit IgG conjugated to horseradish peroxidase (Life Technologies). Blots were reprobed with antibody against β-actin (Santa Cruz Biotechnology) for normalization. Enhanced chemiluminescence was used for detection (Millipore) following manufacturer's instructions. Immunoreactive proteins were visualized with ChemiDoc XRS (Bio-Rad, Marnes-la-Coquette, France) and densitometric analysis was performed with Quantity One® 1-D analysis software (Bio-Rad).

Results

AcSDKP stimulates the growth of human keratinocytes and fibroblasts

AcSDKP used at three different concentrations enhanced the proliferation rate of HaCaT and NHDF cells. As shown in Fig. 1, 3-day treatment with AcSDKP tested at 10^{-7} M, 10^{-9} M and 10^{-11} M resulted, respectively, in 43%, 41% and 55% increase in the proliferation of HaCaT cells as compared with the vehicle-treated control. The similar stimulation of proliferative activity was observed for AcSDKP-treated NHDF cells (15%, 18% and 24% increase in proliferation level observed, respectively, in the presence of 10^{-7} M, 10^{-9} M and 10^{-11} M AcSDKP). As shown, 10^{-11} M AcSDKP yielded maximal stimulation. These results clearly evidence a significant growth activating effect of AcSDKP on two main cellular components of the skin.

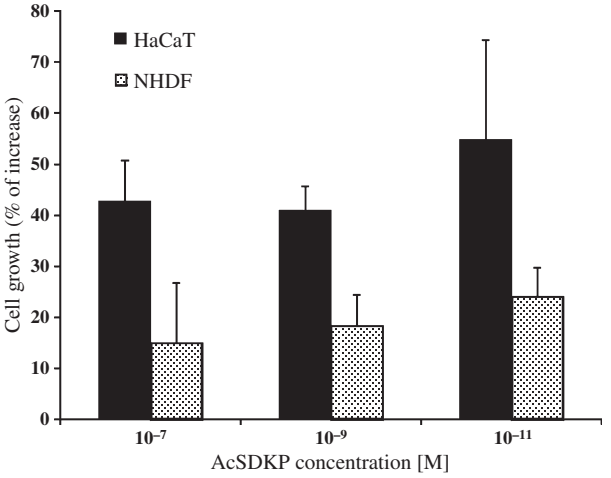

Figure 1 Effect of AcSDKP on the proliferation of human HaCaT keratinocytes and dermal NHDF fibroblasts. Each value represents the mean ± SEM of three independent experiments.

AcSDKP stimulates in vitro the growth of the human interfollicular epidermal basal layer cells as determined by a colony formation assay

Basal keratinocytes of the human interfollicular epidermis constitute a heterogeneous cellular population. Being involved in regenerative processes of wound healing, these cells in the physiological conditions ensure the permanent renewal of the normal epidermis [24, 25]. To determine the effect of AcSDKP on the proliferative capacity of epidermal basal keratinocytes, an *in vitro* clonogenic assay revealing their capacity to form colonies was carried out. As shown in Fig. 2, a slight but statistically significant increase in the number of formed colonies corresponding to KSC (16% and 18%) and progenitors (14% and 16%) was evidenced following treatment with AcSDKP tested at 10^{-5} M and 10^{-10} M. Interestingly, this effect was more important for the lower concentration of AcSDKP (10^{-10} M). These results provide evidence of a growth stimulatory effect of AcSDKP on human epidermal KSC and progenitors.

Topically administered AcSDKP exhibits anti-ageing effect on human skin explants

To assess the direct effect of AcSDKP on human skin, we used an e*x vivo* skin-explant model. The full-thickness skin explants remain viable in culture for at least 2 weeks and consist of both epidermis and dermis. The explants contain all cell types resident in the skin and therefore they are the appropriate models to assess the efficacy of new cosmetic ingredients.

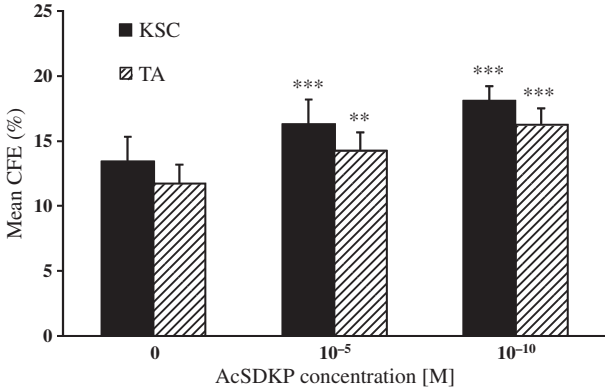

Figure 2 Effect of AcSDKP on the growth of human epidermal keratinocyte stem cells (KSC) and keratinocyte progenitors (TA). Three experiments were carried out using independent foreskin samples. Data are the mean ± SEM values. Statistical significance is indicated as ***$P < 0.001$ or **$P < 0.01$ compared with control condition.

As compared with the control explants treated with saline, the topical application of 10^{-5} M AcSDKP increases significantly the epidermis thickness already after 6 days of treatment (Fig. 3A). The simultaneous improvement in the expression of CK14 and CK19 was noted. The immunostaining of CK14 showed a gradual increase in the level of this protein in basal and suprabasal position (Fig. 3B). The parallel immunostaining of CK19 revealed its up-expression in the skin dermal annexes (Fig. 3C). An important increase in the amount of collagen III in the papillary dermis along the dermo-epidermal junction and more moderate increase in collagen IV were also observed (Fig. 3D,E). Immunolabeling of fibronectin showed that AcSDKP strongly upregulates the expression of this protein along the dermo-epidermal junction and in the papillary and reticular dermis of treated skin explants (Fig. 3F). Staining of histological sections of AcSDKP-treated skin explants with alcian blue-PAS revealed a significant increase in the expression of acid GAGs essentially composed of hyaluronic acid. This effect was characterized by an increased number of PAS-positive fibroblasts in the papillary dermis (especially in a band along the dermo-epidermal junction) and by an increase in GAGs expression in these cells (Fig. 3G).

All these effects were noted already after 6 days of treatment with both 10^{-8} M and 10^{-5} M AcSDKP. However, the efficacy of 10^{-8} M AcSDKP (data not shown) was slightly weaker than this reported for 10^{-5} M AcSDKP. The observed molecular modifications correspond to the criteria for a younger skin and are likely to contribute to the restructuring, regenerating and firming action of AcSDKP.

AcSDKP modulates human hair growth *in vitro* and *ex vivo*

Hair dermal papilla cells are specialized mesenchymal cells that exist in the dermal papilla located at the bottom of hair follicles. These cells play pivotal roles in hair formation, growth and cycling [26]. Therefore, the most useful and rapid method for evaluating hair growth promotion is the *in vitro* assessment of HFDPC growth. As shown in Fig. 4(A), a constant 10% increase in the number of HFDPC cells was observed after 3 days treatment with AcSDKP at three different concentrations.

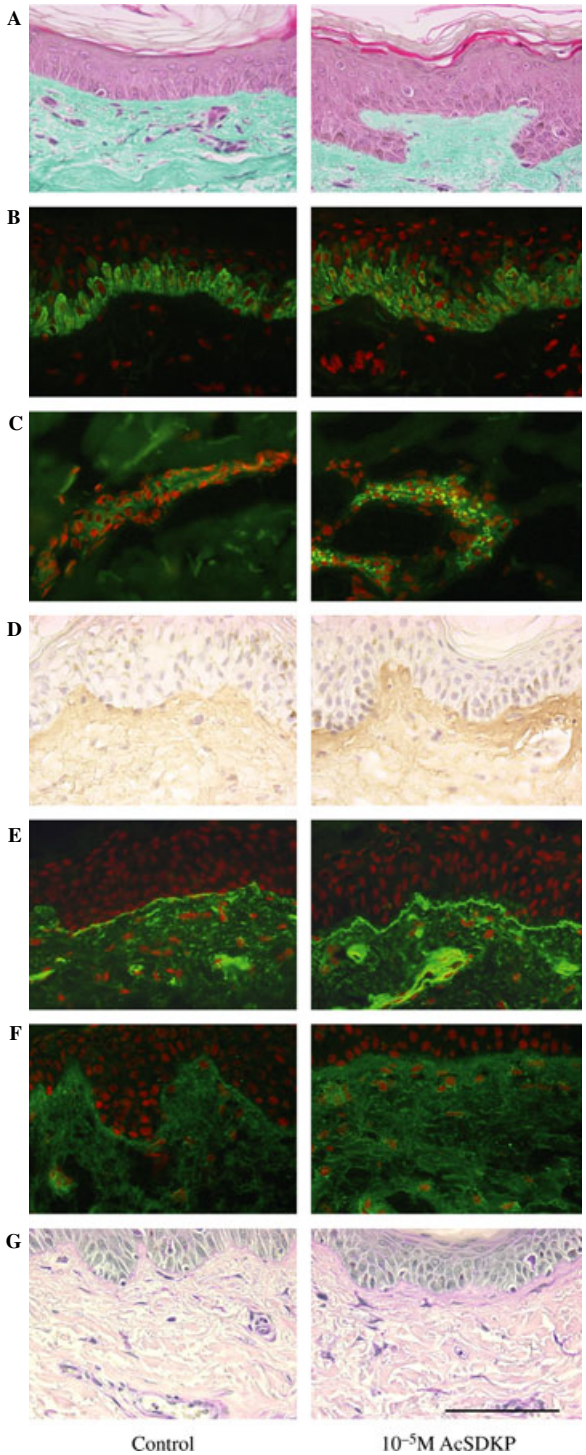

Figure 3 Effect of AcSDKP on skin morphology and expression of skin molecular components using *ex vivo* human skin explants model. All explants were treated every 2 days with 10^{-5} M AcSDKP. The thickness of epidermis (A), expession of CK14 (B), CK19 (C) collagen IV (D), collagen III (E), fibronectin (F) and GAGs (G) were evaluated following 6 days of treatment with AcSDKP. Scale bar = 100 μm.

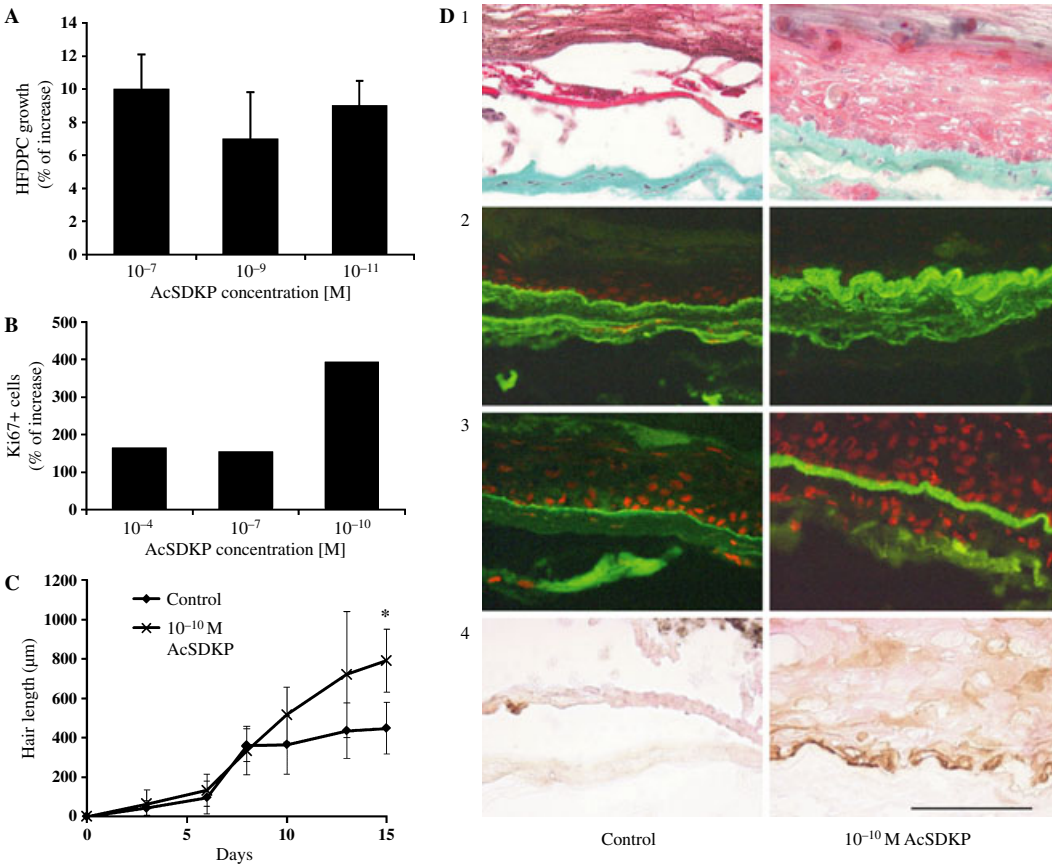

Figure 4 Hair growth promoting effects of AcSDKP (A) *in vitro* stimulation of HFDPC growth; the results are the mean values ± SEM of triplicate measurements. (B) *ex vivo* increase in the number of Ki67$^+$ cells evaluated at day 8 of hair follicle treatment (C) *ex vivo* air shaft elongation; changes in hair length were calculated from the photographs and expressed as mean ± SEM of the lengths of six hairs. (D) histological analysis of control and AcSDKP-treated follicle: (1) general morphology (2) collagen IV (3) laminin 5 (4) CK19. Scale bar = 100 μm. Statistical significance is indicated as *$P < 0.05$ (ANOVA test)

This *in vitro* stimulatory activity of AcSDKP is in line with the *ex vivo* observed significant increase in mitotic index of epithelial cells distributed along the outer epithelial sheath of the follicle, a region within stem cells reside when the hair is in growth anagen phase (Fig. 4B). The number of Ki67$^+$ cells was shown to increase, respectively, 150%, 149% and 400% following 8-day treatment with 10^{-4}, 10^{-7} and 10^{-10} M AcSDKP. These findings correlate with a subtle but distinct elongation of hair shafts. As shown in Fig. 4(C), the treatment of *ex vivo* cultured human hair follicles with 10^{-10} M AcSDKP for 15 days resulted in significant increase in hair shaft length as compared with vehicle-treated follicles.

The further histological investigations revealed the significant restructuration of the epithelial sheaths of hair follicles treated with 10^{-10} M AcSDKP. As shown in Fig. 4(D), this effect well visible at day 15 of treatment, results both in the appearance of the neokeratinocytes along the entire length of the follicle from the epidermis to the bulb and in enhanced synthesis of type IV collagen and laminin 5 in the bulb, especially in the middle part of follicle between the bulge and the bulb. The simultaneous increase in the expression of keratin 19 recognized as one of the epidermal stem-cell markers and distributed along the outer sheath of the follicle strongly suggests epithelial stem-cell activation. These data hint that AcSDKP holds potential for treatment of hair loss and age-related scalp deterioration.

AcSDKP increases the expression of collagen I in human dermal fibroblasts

The treatment of NHDF cells with AcSDKP at three different concentrations for 24 h showed the enhancement of collagen I synthesis. As visualized by immunocytochemistry, all AcSDKP-treated NHDF cells express higher level of collagen I than the vehicle-treated controls. The best response was noted for the fibroblasts treated for 24 h with 10^{-9} M AcSDKP (Fig. 5).

Upregulation of tight junction proteins in human keratinocytes treated with AcSDKP

The expression of three major structural components of tight junctions (TJ) in the human keratinocytes was firstly evaluated by immunocytochemistry. As shown in Fig. 6(A), a marked increase in the expression of ZO-1, claudin-1 and occludin as well as their altered distribution were visualized in human HaCaT keratinocytes treated for 24 h with AcSDKP at 10^{-9} M.

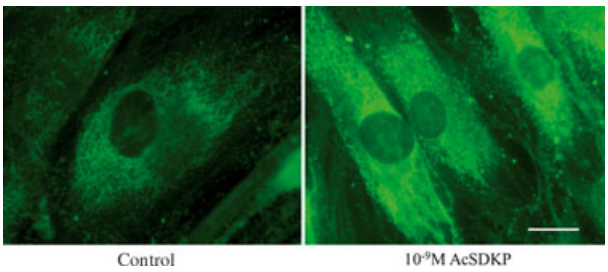

Figure 5 Immunofluorescence analysis of collagen I expression in control and 10^{-9} M AcSDKP-treated NHDF cells. Scale bar = 20 μm.

These data were next validated by Western blot analysis of TJ proteins present in extracts of HaCaT keratinocytes treated with AcSDKP (Fig. 6B). As shown in Fig. 6(C), ZO-1 expression was increased respectively by 66%, 41% and 7% in HaCaT cells exposed to 10^{-7}, 10^{-9} and 10^{-11} M AcSDKP when compared to the vehicle-treated control. Similarly, analysis of cell lysates from AcSDKP treated HaCaT cells showed the upregulation of claudin-1 by 57%, 46% and 23% and occludin by 10%, 37% and 62%, respectively, for 10^{-7}, 10^{-9} and 10^{-11} M AcSDKP.

These findings suggest that the altered TJ protein expression induced by AcSDKP may lead to better control of skin permeability with improved hydration.

AcSDKP upregulates SIRT1 expression in normal human keratinocytes and fibroblasts

The effect of AcSDKP on the expression of SIRT1 protein was examined *in vitro* using HaCaT and NDHF cells. SIRT1 expression was investigated by immunostaining and next quantified by Western blotting. As shown in Figs 7 and 8, AcSDKP significantly upregulated the synthesis of SIRT1 in both types of skin cells. The results of Western blot analysis presented in Fig. 7(B,C) revealed an average 200% increase in SIRT1 expression in the human keratinocytes treated for 24 h with AcSDKP at three different concentrations (10^{-7} M, 10^{-9} M and 10^{-11} M). The extent of the AcSDKP effect was similar to that found for treatment with 10 μM resveratrol.

Discussion

Short sequence peptides ranging from 2 to 20 amino acids represent a relatively new and promising set of additives for the skincare industry [8–10]. Indeed, peptides play a central role in the regulation and modulation of biological responses that occur in nature. Recent studies suggest that treatment with various biologically active peptides can result in favourable changes in collagen, elastin and melanin synthesis [10, 27].

In this study, we show that the ubiquitous tetrapeptide AcSDKP widely present in mammals and recently detected at micromolar concentration in the aqueous extracts of shiitake (data not shown)

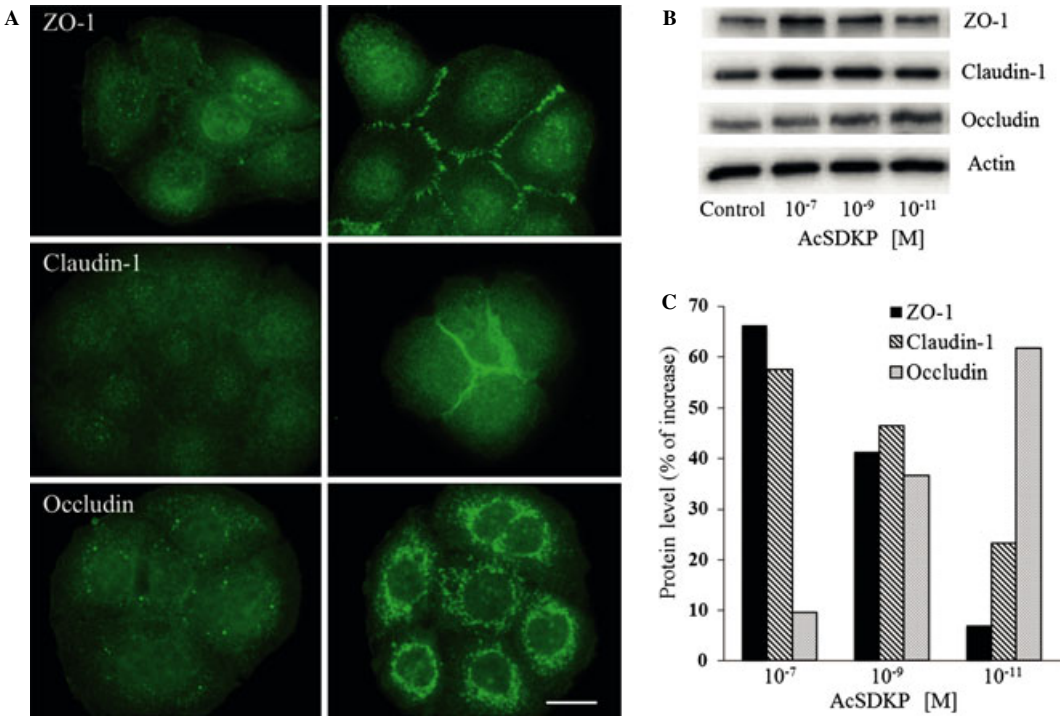

Figure 6 AcSDKP increases expression of tight junction proteins in human HaCaT keratinocytes. (A) Immunocytochemical staining of ZO-1, claudin-1 and occludin in HaCaT cells treated with AcSDKP for 24 h. Scale bar = 20 μm. (B) Western blot analysis of ZO-1, claudin-1 and occludin from cell lysates of AcSDKP-treated HaCaT cells (C) relative expression ratio of the bands presented on panels (B). Differences in loading were adjusted to actin protein level.

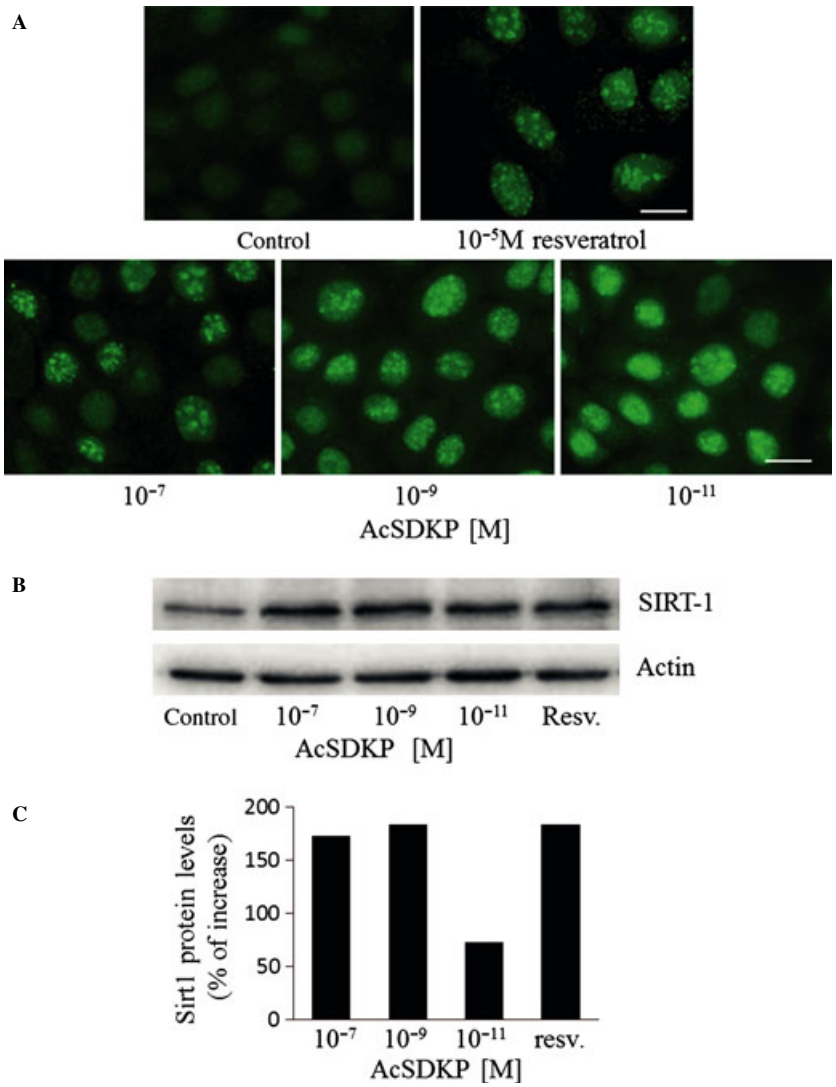

Figure 7 SIRT1 expression is upregulated by AcSDKP (A) Immunocytochemical staining of SIRT1 in human HaCaT keratinocytes. Scale bars = 20 μm (B) Western blot analysis of SIRT1 level in HaCaT cells; actin was used as a loading control (C) relative expression ratio of the bands presented on panels B.

regulates a number of molecular/cellular events related to skin and hair ageing. AcSDKP is a patented anti-ageing natural tetrapeptide resulting from our latest research in the area of skin and hair protection [28, 29]. The current studies reported in this article were undertaken after the discovery of proangiogenic potency of AcSDKP [13, 14]. The important role of the blood supply in controlling skin and hair biology has long been known. Likewise, the impairment of cutaneous vasculature is one of the consequences of skin ageing [17]. Therefore, the initial strategy in skin rejuvenation should be to improve the cutaneous vascular integrity because this will allow the skin to react more effectively to other forms of anti-ageing treatment. In this regard, it is noteworthy that AcSDKP appears to be an excellent candidate for the improvement of the blood supply when it is applied to the skin.

On the other hand, it is well-known that improved follicle vascularization promotes hair growth and increases hair follicle and hair size. Indeed, the vascular network is tightly associated with the hair growth cycle [18, 30].

The importance of angiogenesis in hair growth stimulation has been further supported by the study on the activity of minoxidil which upregulates the expression of VEGF in normal human hair follicles, thereby promoting the conservation of vascularization of hair dermal papilla, which may contribute to the extension of the anagen phase in androgenetic alopecia [31]. AcSDKP could thus help promote hair growth in both men and women with poor scalp micro-circulation and follicle atrophy caused by ageing.

Skin ageing is characterized by a progressive deterioration of its functional properties, linked to alterations of epidermis and dermal connective tissue [32, 33]. Epidermal ageing includes mainly epidermal thinning. In fact, the proliferative rate of keratinocytes in the basal layer of the epidermis slows down and the replacement of the dead cells which are continuously shed from the surface is

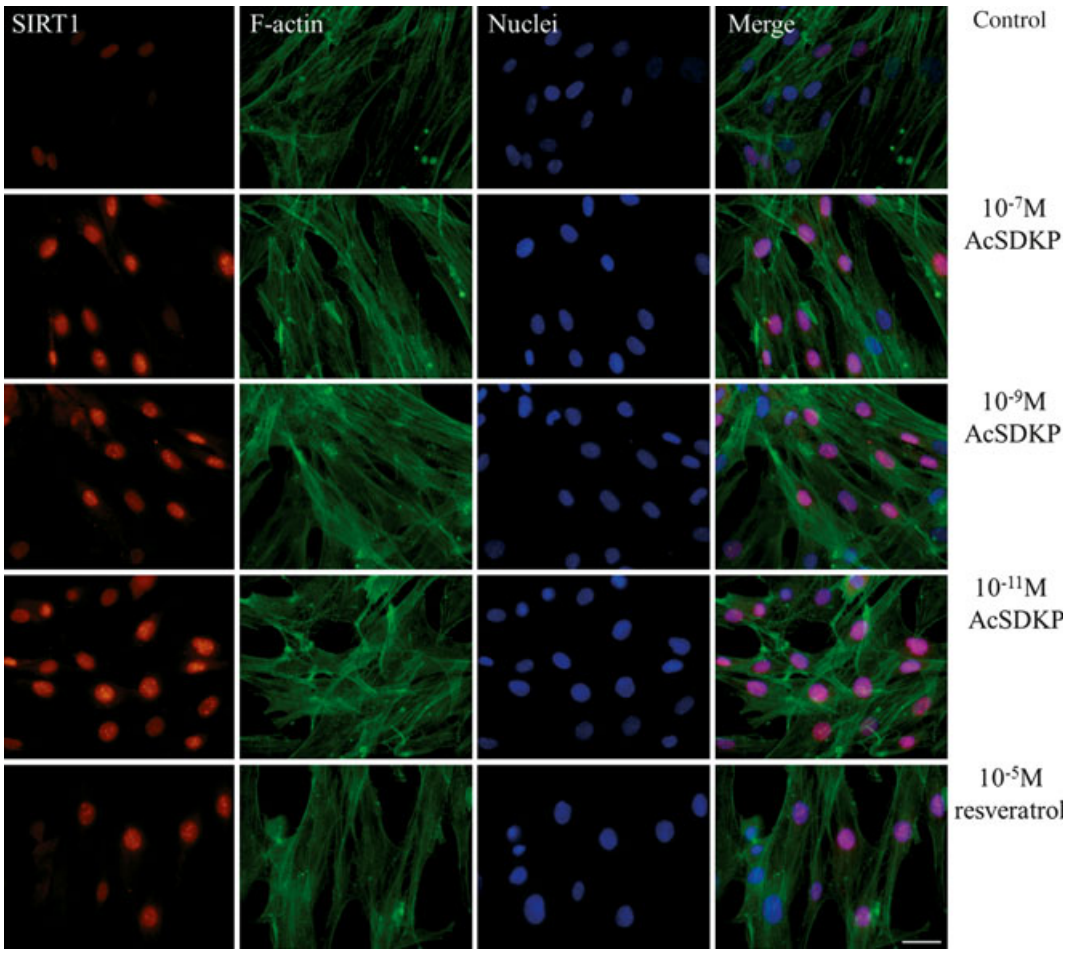

Figure 8 SIRT1 expression in NHDF fibroblasts is upregulated by AcSDKP. Scale bar = 20 μm.

impaired. Moreover, this decreased growth of keratinocytes producing the components of the dermal-epidermal junction is impaired and the adherence between the two major skin layers is modified. The effect of ageing on the dermis, which incorporates various cell types into a complex extracellular matrix composed mainly of collagen, is also significantly changed. Not only does the dermal layer thin as a result of a decrease in fibroblast numbers, but also the consequent synthesis of various collagens and elastin is reduced resulting in the loss of the three-dimensional integrity of the skin and in the development of wrinkles [34]. The impairment of dermal proteoglycans and glycosaminoglycans (GAG) synthesis composed largely of water-holding sugar moieties makes the ageing skin dryer and looser [35, 36].

Using the *ex vivo* model of cultured human skin explants, AcSDKP at a concentration of 10^{-5} M was shown to reduce the signs of skin ageing by increasing epidermal proliferation to restore epidermal thickness by 6 days of treatment. Moreover, the consequent increase in the synthesis of keratins 14 and 19 in epidermis is in line with the improved control of the epidermal barrier. These findings are correlated with the significant increase in keratinocytes growth resulting from AcSDKP *in vitro* treatment. Similar to the dose-response observed for induction of angiogenesis [13], AcSDKP at 10^{-11} M yielded maximal stimulation.

As the induction of collagen in ageing skin is essential for recovery of skin firmness and knowing that loss of proliferation is a typical characteristic of senescence, we believe that the successful stimulation of human fibroblast growth by AcSDKP and the enhanced production of various collagens observed both *in vitro* and *ex vivo* experiments are relevant to the improvement of human skin elasticity. Indeed, the higher expression of collagen I in cultured fibroblasts, together with the enhanced expression of dermal collagens III and IV in human skin explants treated with AcSDKP, should restore age-induced dermal thinning and improve skin appearance as it was already demonstrated for topically applied pentapeptide KTTKS [37] and tetrapeptide GEKG [38]. The simultaneous *ex vivo* promotion of fibronectin synthesis should also contribute to the AcSDKP rejuvenating effect as it was shown previously for the bioactive pentapeptide subfragment of type I collagen [39]. The reported increase in GAGs synthesis in skin explants treated with AcSDKP also supports the compensatory role of this peptide for age-related skin dehydration. Indeed, the hyaluronic acid, which represents approximately 70% of the GAGs, is

known to play a key role in the preservation of tissue hydration [40].

Cutaneous hydration is also regulated by the mechanism related to the control of water loss. The upper layer of the epidermis made up of keratinocytes sealed by tight junctions (TJs) acts as a waterproof-protective barrier preventing skin dehydration. TJs are cell-cell adhesive zipper-like structures which prevent diffusion of solutes through the intercellular spaces and protect the skin from unnecessary water loss as well as the entry of detrimental influences from the outside [41]. A variety of TJ proteins including claudins, occludin, zonula occludens-proteins (ZO) and junctional-adhesion molecules have been identified in mammalian epidermis [42]. Transepidermal water loss (TEWL) depends thus on the capacity of keratinocytes to synthesize the proteins building tight junctions. Indeed, extensive epidermal water loss was observed in claudin-1-deficient mice [43].

Currently, elderly people are more likely to suffer from dry skin than young, healthy individuals. This is because of the impaired skin barrier and accelerated TEWL linked to the extensive remodelling of the TJ occurring with age [33]. The skin, once dehydrated, becomes dull, lifeless, non-resilient and more sensitive. Besides the application of products that reduce evaporation from the skin by forming a film impervious to water, the use of actives acting on the internal processes in the skin and restoring the barrier function is currently in practice. The local application of AcSDKP that induces a significant increase in claudin-1, occludin and ZO-1 synthesis is expected to form a barrier which will be more impervious and thus contribute to improved skin hydration.

The recent findings have shed new insights into the function of tight junctions in skin pathology. They provide evidence for an impaired skin barrier in human atopic dermatitis, which is a multifaceted disease characterized by dry skin and a hyperactive immune response to allergens. Indeed, TEWL was shown to correlate with disease severity in humans [44, 45]. Furthermore, it was reported that a deficiency in protein-forming tight junctions contributes to the barrier dysfunction in atopic dermatitis subjects and that this may be mediated in part by reduction in claudin-1 synthesis [46]. Thus, the ability of AcSDKP to enhance the synthesis of claudin-1, as well as two others structural proteins of tight junctions, suggests the therapeutic efficacy of this peptide to improve clinical skin parameters in patients suffering from atopic dermatitis.

The skin constantly renews itself throughout adult life, and the hair follicle undergoes a perpetual cycle of growth and degeneration. Stem cells residing in the epidermis and hair follicle ensure the maintenance of adult-skin homeostasis and hair regeneration. However, when they are ageing their potency to restore the senescent cell compartments is compromised [47]. In fact, it was already reported using keratinocytes from donors of different ages that chronological ageing induced loss of stem cells in the skin [48]. On the other hand, functional depletion of stem cells results in ageing [49]. Thus, the search for compounds that are able to ensure proliferation and functionality of epidermal stem cells is one of the areas emerging in the personal care industry. The slight but significant activation of the epidermal stem cells growth by AcSDKP could thus boost the skins own renewal resources, which get weaker with age and suggest the ability of this peptide to delay skin ageing. Taking into consideration that stem cells in the epidermis contribute to repair the skin after injury, AcSDKP should also accelerate age-impaired skin healing.

Inspired by the latest research on genes influencing cell longevity, sirtuin 1 (SIRT1) has been found to be of importance in cosmetology and represents an interesting target for cosmetic ingredients. Called the 'human longevity protein', SIRT1 plays a key role in the control of the ageing process reducing the incidence of age-related disorders. This protein is a member of a highly conserved gene family (sirtuins) encoding NAD^+-dependent deacetylases, originally found to deacetylate histones leading to increased DNA stability and prolonged survival in mammals cells [50]. A growing body of evidence suggests a role for SIRT1 in maintaining energy and nutrient homeostasis, thereby linking its anti-ageing property to its role in metabolism [51]. Indeed, the variety of SIRT1 substrates suggests that this protein is involved in the regulation of diverse biological processes, including not only cell survival and apoptosis but also gluconeogenesis, adipogenesis, lipolysis, stress resistance and insulin secretion [52, 53]. SIRT1 was reported to extend the replicative lifespan of human cells [54], an effect that can be attributed, at least in part, to the SIRT1-mediated deacetylation and inhibition of p53 [55].

Emerging evidence suggests the involvement of SIRT1 in skin cell senescence. It has been previously shown that SIRT1, which is functionally expressed in human-skin keratinocytes and fibroblasts [56], is downregulated by both UV exposure and H_2O_2, two major inducers of skin cell damage [57]. These skin changes were prevented by resveratrol, suggesting that SIRT1 activators may protect skin against the adverse effects of solar UV radiation increasing cellular viability, and diminishing DNA damage. Moreover, it was reported that enforced SIRT1 expression promoted cell proliferation and antagonized cellular senescence in human fibroblasts [58].

However, the cellular level of SIRT1 decreases with age. Thereby the search for activators of the activity and/or synthesis of this enzyme as candidates for the role of an anti-ageing factor is clearly of importance. The demonstrated ability of AcSDKP to boost significantly the synthesis of SIRT1 as well as its enzymatic activity may thus contribute to both skin and hair cell rejuvenation. Indeed, transgenic animals characterized by reduced levels of SIRT1 have a phenotype of accelerated ageing and exhibit several features of premature ageing of the skin, including hair loss because of the reduction in hair follicles [59]. An important increase in the synthesis of the longevity-related protein, SIRT1, mediated by AcSDKP may also contribute to the protection of the skin against damage caused by UV radiation. As it was shown previously, the clinical evaluation of a formulation enriched in the yeast Kluyveromyces biopeptides that increase SIRT1 expression in human skin, revealed the efficacy of this ingredient in treating multiple skin-ageing signs such as fine lines and wrinkles, hydration, pigmented spot colour intensity, complexion radiance, firmness, complexion homogeneity and texture [60].

It is known that SIRT1 activation also results in inhibition of the inflammatory pathways in a variety of cell types including fibroblasts and macrophages [61, 62]. The fact that AcSDKP upregulates the synthesis of SIRT1 leads us to expect that this peptide may have significant anti-inflammatory properties. In fact, age-associated inflammatory processes, known as 'inflamm-ageing' contributes largely to the progressive degradation of skin structures [63].

Therefore, the peptides which reduce the level of proinflammatory cytokines were shown to produce increased skin firmness, smoothness and elasticity [64]. Therefore, the previously reported reduction of renal and cardiac inflammation by AcSDKP [65, 66] together with the current findings strongly support the view that AcSDKP is a novel anti-ageing protein involved both in the regulation of cellular senescence and inflammation.

Applying all this new data related to the AcSDKP ability to boost *in vitro* and *ex vivo* growth of skin cells as well as to upregulate the expression of their structural components undergoing extensive deterioration with age, we have evaluated its benefits on human scalp.

The appearance of hair plays an important role in peoples' overall physical look and self-perception. The haircare industry has become aware of this, and constantly search to deliver active products that are directed towards meeting this consumer demand. The appearance and function of the scalp and hair shaft can be affected by age in the same manner as skin. Hair porosity, elasticity and texture change; the hair becomes thinner. Moreover, most older adults are affected by some scalp hair loss as part of the ageing process. Hair growth and renewal are determined mainly by the activity of the hair follicle and the surrounding dermo-epidermal environment. Taking into account the efficacy of AcSDKP to stimulate the growth of fibroblasts and keratinocytes, one might suspect that AcSDKP incorporated in the haircare formulation will penetrate the hair shaft and scalp whilst creating a regenerative and protective action on the hair. The beneficial effect of factors possessing the keratinocyte growth-promoting activity is well recognized both for hair and skin. In fact, keratinocyte growth factor (KGF), also known as FGF-7 whose mitogenic activity is predominantly exhibited in keratinocytes has been proven to increase skin volume and stimulate hair growth [67]. This behaviour is especially beneficial in preventing hair loss because of the effects of ageing or the side effects of chemotherapy. Moreover, an increase in interfollicular keratinocyte proliferation could also prevent hair loss. In fact, the reduced proliferative rate of the epidermal cells was shown to be associated with entry into the regressive phase of the hair follicle cycle (catagen), which occurred earlier than usual [68].

The successful *in vitro* stimulation of HFDPC by AcSDKP at nanomolar concentration contributes to the beneficial effects of this peptide on hair growth. It must be emphasized that HFDPC cells being implicated in hair follicle development and growth are thought to be a reservoir of multi-potent stem cells [69]. Indeed, we have observed four-fold increase in mitotic index of follicle epithelial cells characterized by increased expression of keratin 19 (a marker of stem cells) following 1-week treatment of scalp explants with AcSDKP. The consequent hair elongation revealed in *ex vivo* studies together with AcSDKP restructuring effect on the epithelial sheaths of hair follicles (enhanced synthesis of various structural proteins of the hair follicle) should improve the quality of the hair shaft and slow hair loss in elderly. An important increase in the synthesis of SIRT1 mediated by AcSDKP in the keratinocytes and fibroblasts may also contribute to hair cell rejuvenation.

All our findings reported in this article are consistent with previously documented skin-repairing properties of thymosin β 4 (Tβ4) and suggest that AcSDKP and its molecular precursor are physiologically relevant regulators of pathways controlling cell ageing. In fact, AcSDKP characterized by its potent angiogenic activity and wound healing potency is generated in the body from the polypeptide of 43 amino acids, Tβ4, recognized also as angiogenic factor and thus implicated in the process of wound healing [70]. Tβ4 is believed to help rejuvenate ageing skin. Ultraviolet radiation damage or other injuries to skin that are associated with ageing may be in the future repairable with Tβ4, similar to the success with wound repair. Ongoing research demonstrates the powerful effect of Tβ4 on ageing skin, in part because of its ability to stimulate the production of collagen and elastin [71]. Moreover, the well-documented ability of Tβ4 to enhance hair growth stay in line with the reported properties of AcSDKP [72, 73].

Greater understanding of the mechanisms of ageing and the molecular disorders within skin and hair follicle cells are leading to new therapeutics to treat not only skin/scalp disease and injury but also their aesthetic appearance. We report here the potency of a naturally occurring tetrapeptide AcSDKP to compensate for these molecular ageing disorders.

In summary, AcSDKP was shown to exert the potential anti-ageing benefits via improved epidermal proliferation and an enhancement of synthesis of macromolecular components of the epidermis and dermis which are essential for the maintenance of young and healthy skin. Supporting the tight junction, AcSDKP helps to strengthen the skin's barrier function what could provide better hydration of the epidermis. An important increase in the synthesis of a longevity-related protein, SIRT1, mediated by AcSDKP coupled with growth stimulatory effect of this peptide on epidermal keratinocyte progenitor and stem cells will also contribute to skin and hair cell rejuvenation. The further morphological and molecular modifications of the hair follicles induced by AcSDKP matching the criteria of hair growth support the use of AcSDKP to prevent hair deterioration caused by age.

Considering the cosmetically interesting effects of AcSDKP reported in this article, the ability of this peptide to contribute to skin and hair functional and structural recovery, and to slow down the premature entry into cell senescence needs now to be validated by *in vivo* tests on human volunteers.

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