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A secretion of the mollusc *Cryptomphalus aspersa* promotes proliferation, migration and survival of keratinocytes and dermal fibroblasts *in vitro*

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Synopsis

Regenerative properties of skin decrease with age, and thus, the search for substances that minimize cutaneous ageing has increased in the last few years. The secretion of the mollusc *Cryptomphalus Aspersa* (SCA) is a natural product that bears regenerative properties when applied topically. The purpose of this work is to study the *in vitro* effects of SCA on cell proliferation and migration, as well as on cell–cell (E-cadherin and β -catenin) and cell–substrate (vinculin and β 1-integrin) adhesion proteins expression, using a human keratinocyte cell line (HaCaT cells) and primary dermal fibroblasts (HF). We tested the effects of SCA on cell proliferation using a colorimetric assay. In addition, SCA-induced changes on cell migration were studied by wound-healing assays. Besides, Western blot and immunofluorescence microscopy were carried out to test the expression of different cell adhesion proteins. We found that SCA promotes proliferation and migration of HaCaT cells in a time- and dose-dependent manner. Moreover, treatment with SCA increases the migratory behaviour and the expression of adhesion molecules in both HaCaT and HF. Finally, SCA also improves cell survival and promotes phosphorylation of FAK and nuclear localization of β -catenin. These results shed light on the molecular mechanisms underlying the regenerative properties of SCA, based on its promoting effect on skin cell migration, proliferation and survival. Moreover, these results support future clinical uses of SCA in the regeneration of wounded tissues.

Introduction

Skin degeneration is a complex process induced by ageing and environmental damage, for example UV light. When considering the skin, UV light is the most important damaging factor, leading to skin photoageing and cancer. The main mechanism of UV-induced photoageing is a marked increase in the production of reactive oxygen species (ROS), which causes photo-oxidative damage and decreases cell migration and proliferation [1, 2]. Photoageing is characterized by the appearance of wrinkles, loss of

structural integrity and impaired wound healing [3]. These effects are closely related with alterations in the remodelling process of the extracellular matrix (ECM), a task carried out by dermal fibroblasts [4, 5].

When the skin is injured by any external aggression, the machinery to repair the damage is rapidly activated, including fibroblast proliferation and migration to the wound site to secrete and assemble new ECM components (i.e. collagen, elastin and fibronectin) that renew elasticity and consistency of the skin. In healthy skin, the basal keratinocyte layer attaches to a carpet of specialized matrix, the basal lamina. Therefore, physiological regenerative events include proliferation of keratinocytes and the establishment of new cell–cell and cell–substrate (basal lamina) adhesions. One of the most important molecules involved in intercellular attachment is E-cadherin (ECD), a transmembrane protein that mediates epithelial cell–cell adhesion through other intracellular anchor proteins such as β -catenin in adhesion junctions [6]. Keratinocytes also establish hemidesmosomes, which anchors to the laminin in the basal lamina through specific integrins belonging to the β 1 subfamily [7]. In these cells, hemidesmosomes connect the ECM to the intermediate filament cytoskeleton (cytokeratins) inside the cell. These interactions are crucial not only for the integrity of the skin but also for signal transduction leading to cell migration and proliferation, for example phosphorylation of the Tyr kinase focal adhesion kinase (FAK) [7, 8]. Finally, survival signals are also needed to maintain the integrity of the new repaired tissue. In this sense, elevated levels of nuclear β -catenin also have been associated with improved cell survival [9]. Among the plethora of signals involved in cellular proliferation, β -catenin translocation to the nucleus plays a pivotal role. In the nucleus, β -catenin is incorporated to the transcriptional machinery, stimulating the expression of proliferation-inducing gene products [10].

Research of natural substances that can improve and stimulate skin regeneration has increased in the last few years. In this regard, the secretion of some snails has proven beneficial in the regeneration of burnt skin [11] and in the management of open wounds [12]. In particular, a secretion from the mollusc *Cryptomphalus aspersa* (SCA) has been proven to have antioxidant activity [13] and to induce skin regeneration after wound-healing impairment from acute radiodermatitis [14]. Moreover, a recent study demonstrated that SCA stimulates fibroblast proliferation,

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rearrangement of the actin cytoskeleton and stimulates ECM assembly [15]. However, the molecular mechanisms underlying the regenerative properties of SCA are not completely understood. In the present study, we have investigated the regenerative properties of SCA using different *in vitro* approaches. We found that SCA increased fibroblast and keratinocyte migration and increased the expression of cell–cell and cell–substrate adhesion molecules in both cell types. Finally, we demonstrated a role for SCA in cell survival signalling pathways, because it induces the expression of molecules related with this process, such as active β -catenin, FAK and phosphorylated FAK. Taken together, our results support the use of SCA as a putative cutaneous regenerative product.

Material and methods

SCA preparation

SCA was prepared according to US patent US 5538740. Briefly, the gastropod was physically stimulated by centrifugation to increase the secretion naturally produced by the mucinous, albuminous and salivary glands. Then, the secreted fluids were separated and collected from the live gastropod, clarified by centrifugation and further clarified by filtration through 0.22- μ m filters. Further dilutions were performed in aqueous solution (pH 7.4). SCA toxicity was assayed by trypan blue exclusion method. The highest effect/toxicity ratio was achieved at 100 μ g mL⁻¹ SCA; thus, we employed this concentration for most of the assays described.

Cell cultures

The cells included in the study are HaCaT cells, an immortalized human keratinocytes cell line and human dermal adult fibroblasts (NHDF Cambrex, Charles City, IA, U.S.A.). Cells were grown in F-25 flasks, or 35-mm culture dishes (Costar, Corning, NY, U.S.A.) or on 22-mm square glass coverslips placed into dishes, using Dulbecco's modified Eagle's medium containing 10% (v/v) foetal calf serum, 50 U mL⁻¹ penicillin, 50 mg mL⁻¹ streptomycin and 1% (v/v) 0.2 M L-glutamine (all from Gibco, Paisley, Scotland, U.K.). Cells were incubated at 37°C in an atmosphere containing 5% CO₂.

MTT viability assay

Cell viability was documented by the MTT assay [16]. HaCaT cells cultured on 6-well Petri dishes were treated with different concentrations of SCA (0, 25, 50 and 100 μ g mL⁻¹) during several time points (2, 4, 6 and 8 days). Following these incubations, 3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyltetrazolium-bromide (MTT) solution was added to each well at a concentration of 0.5 ng mL⁻¹, and plates were incubated at 37°C for 2–3 h. The resulting formazan crystals were dissolved by the addition of Dimethyl sulfoxide (DMSO), and absorbance was measured at 560 nm. The viability was also established by microscopy with a trypan blue exclusion test using a Neubauer chamber counter. In both cases, similar results were obtained.

Migration (wound-healing) assays

In vitro wound-healing assays were performed as previously described [15]. Briefly, HaCaT and NHDF cells were seeded in T6-well culture dishes at a density of 3×10^5 cells per well. Confluent cell monolayers were then gently scratched with a pipette tip

across the entire diameter of the dish. Next, cells were rinsed with medium to remove all cellular debris, and treatment with SCA was applied to three wells, whereas the rest of the wells served as control. Cultures were observed immediately after wounding and again 12 and 24 h later, and phase-contrast pictures were taken of the wounded area using a high-resolution microscopic camera Axio-cam MRm vers.3 FireWire (D) (Zeiss, Le Pecq, France) connected to the microscope. Migrated cells were quantified by measuring the healed area. Data are given as means $\pm$ SD from three independent experiments. Statistical significance was evaluated using the Student's *t*-test, and differences were considered to be significant at a value of $P < 0.05$ (*).

Immunostaining

For general immunodetection, cells grown on coverslips were fixed in 3% paraformaldehyde for 15 min. Samples were rehydrated in PBS, permeabilized with 1% Triton X-100 in PBS for 10 min and incubated for 1 h at 37°C with the primary mouse monoclonal antibodies against ECD, β -catenin, vinculin, FAK and phospho-FAK (BD Transduction Laboratories, Palo Alto, CA, U.S.A.) and active β -catenin (Millipore, Billerica, MA, U.S.A.) at 1 : 50 dilution in 0.1% BSA (Santa Cruz Biotechnologies, Santa Cruz, CA, U.S.A.). Afterwards, coverslips were washed with PBS, incubated with rabbit anti-IgG of FITC-labelled secondary antibody (1 : 500 dilution in 0.1% BSA; Santa Cruz Biotechnologies) and finally washed with PBS. The preparations were mounted with Prolong (Invitrogen, Carlsbad, CA, U.S.A.). Microscopic observations and photographs were performed in an Olympus photomicroscope BX61, equipped with a HBO 100 W mercury lamp and the corresponding filter sets for fluorescence microscopy: blue (450–490, exciting filter BP 490) and green (545 nm, exciting filter BP 545). The fluorescence intensity of nuclear active β -catenin was measured using IMAGEJ 1.37v software (<http://rsb.info.nih.gov/ij/>); at least 100 nuclei were counted for untreated (controls) and treated SCA cells, and the values are given as means $\pm$ SD.

Western Blot analysis

Semiconfluent monolayers were washed and then lysed with RIPA buffer (150 mM NaCl, 1% Triton X-100, 0.05% deoxycholate, 0.1% SDS, 1% Non idet-40, 50 mM Tris, pH 8) containing phosphatase cocktail 2 and protease inhibitor cocktail (Sigma, St. Louis, MI, U.S.A.). The samples were adjusted to the same protein concentration (BCA Protein Assay Reagent; Pierce, Rockford, IL, U.S.A.) and denatured by boiling in Laemmli sample buffer with 5% β -mercaptoethanol. 20 μ g of each sample was subjected to electrophoresis separation in SDS-PAGE. Gels were then transferred to a PDVF Immobilon-P membrane (Millipore, Bedford, MA, U.S.A.) for 2 h. Membranes were stained with Ponceau S (Sigma) to control loading, and after destained, they were blocked with 5% skimmed milk in Tris-buffered saline (Tris-HCl 10 mM pH 7.6, NaCl 0.9%, 0.05% Tween 20). The membranes were incubated with the following specific antibodies: anti-ECD, vinculin, FAK, P-FAK (BD Transduction Laboratories), active β -catenin (Millipore) and G-actin (Sigma), diluted 1 : 500 in blocking buffer at 4°C overnight. HRP secondary monoclonal antibody anti-IgG of mouse was used, diluted 1 : 1000 and incubated 2 h at room temperature. Detection of bands was performed using ECL Plus Western blotting detection system (GE Healthcare, Hertfordshire, U.K.). To quantify the bands obtained by Western blot, we applied IMAGEJ software-based analysis.

Results

SCA treatment induces proliferation of HaCaT cells

To test whether SCA affected the proliferation of HaCaT cells, we incubated the cells with different concentrations of SCA for different times (Fig. 1). We found a non-significant increase in cell proliferation compared with control cells at $25 \mu\text{g mL}^{-1}$, whereas higher concentrations of SCA (50 or $100 \mu\text{g mL}^{-1}$) induced a gradual and significant increase with the time of treatment (Fig. 1).

SCA treatment stimulates migration of HaCaT cells and human fibroblasts

We evaluated whether exogenous treatment with SCA had an effect on the migration of keratinocytes and fibroblasts, using a classical wound-healing assay. Confluent cultures of HaCaT cells and human fibroblasts were gently scratched with a pipette tip and treated with $100 \mu\text{g mL}^{-1}$ SCA for 24 h. Photographs were taken before ($t = 0$), and 12 and 24 h after wounding (Fig. 2). As shown in Fig. 2(A,C), SCA promotes rapid occupation of the space between both limits of the wound compared with control, untreated HaCaT cells. At 24 h after SCA addition, the wound was completely occupied with cells, whereas there was still an open space in control cells. Similar results were found with human dermal fibroblasts; however, closure occurred at a slower rate (Fig. 2B,C).

SCA treatment increases the expression of cell–cell and cell–substrate adhesion molecules

We then focused on the expression of molecules that mediates intercellular adhesion and attachment to the substrate. Immunofluorescence and Western blot experiments revealed that treatment of HaCaT cells with $100 \mu\text{g mL}^{-1}$ SCA for 48 h stimulated the expression of both ECD and β -catenin (Fig. 3A). Densitometry analysis of Western blot bands revealed a 3.2-fold increase of ECD expression and, more remarkably, a 28-fold increase for β -catenin (Fig. 3B) with SCA treatment. Moreover, SCA not only increased the expres-

sion of ECD in keratinocytes, but it also seems to contribute to the organization of ECD in cell–cell junctions (Fig. 3A, lower panel).

To study the effect of SCA treatment in cell–matrix adhesion, we analysed the expression and distribution of the focal adhesion marker vinculin in both cultured HaCaT cells and in human fibroblasts with SCA for 48 h ($100 \mu\text{g mL}^{-1}$). As seen in Fig. 4(A), immunofluorescence studies revealed that SCA promotes the recruitment of vinculin to focal adhesions in HaCaT cells, which correlated with increased vinculin expression as determined by Western blot analysis (Fig. 4B). Moreover, we also studied by Western blot the effect of SCA in β 1-integrin production, a very important molecule involved in cell–substrate adhesion. Results from these experiments are shown in the lower panel of Fig. 4B, and it can be seen that SCA treatment induced the expression of vinculin and β 1-integrin in HaCaT cells by 3.5- and 9.5-fold increase, respectively, compared with control, untreated cells. With respect to human fibroblast, we also found an important increase in the expression of β 1-integrin and vinculin mediated by exogenous treatment with SCA, shown by both, immunofluorescence (Fig. 4A) and Western blot analysis (4- and 5.3-fold increase vs. untreated cells, Fig. 4B).

SCA treatment promotes the expression of survival molecules in keratinocytes and human fibroblasts

To assess the effect of SCA treatment on cell survival, we studied the expression of molecules involved in this process, both by immunofluorescence and Western blot. As seen in Fig. 5A (left panels), we found an increase in β -catenin expression in HaCaT cells, when treated with $100 \mu\text{g mL}^{-1}$ SCA for 48 h, compared with untreated cells. Furthermore, distribution of this molecule was predominantly at the nuclear area in SCA-treated cells, whereas in control cells, β -catenin was mainly located at the plasma membrane (Fig. 5A, left panels). The fluorescence intensity because of nuclear β -catenin was notably higher in SCA-treated cells compared with that of controls (14.06 ± 3.28 vs. 1.3 ± 0.42 ; Fig. 5B). Western blot studies also revealed a 13.6-fold increase in protein production of active nuclear β -catenin in SCA-treated vs. control HaCaT cells (Fig. 5C).

We next investigated the expression and activation by phosphorylation of FAK, which is another molecule involved in cell survival, in human fibroblasts treated with SCA $100 \mu\text{g mL}^{-1}$ for 48 h. Immunofluorescence studies show a clear increase in both expression and phosphorylation of FAK when SCA is present, compared with control cells (Fig. 5A, right panels). Comparable results were obtained by Western blot in human fibroblasts (data not shown) and in HaCaT cells (Fig. 5C). In these cells, we found that expression of FAK is increased 5-fold, whereas its phosphorylation increased 24-fold compared with control, untreated cells.

Discussion

Skin ageing is a natural biological process. However, environmental aggression and genetic predisposition can accelerate this process. For example, exposure to UV wavelengths promotes oxidative damage, in a process referred to as photoageing. Photoageing affects the sun-exposed areas and is characterized clinically by wrinkles, roughness, dryness, laxity, telangiectasia, loss of tensile strength and pigmentary changes in the skin. There is also an increase in the development of benign and malignant neoplasms on photoaged skin. Several pathological mechanisms are involved in the process of skin ageing, such as an increase in ROS production [17], impaired remodelling capacity of the skin, reduced cell migration, adhesion and survival [1]. The search for products that can

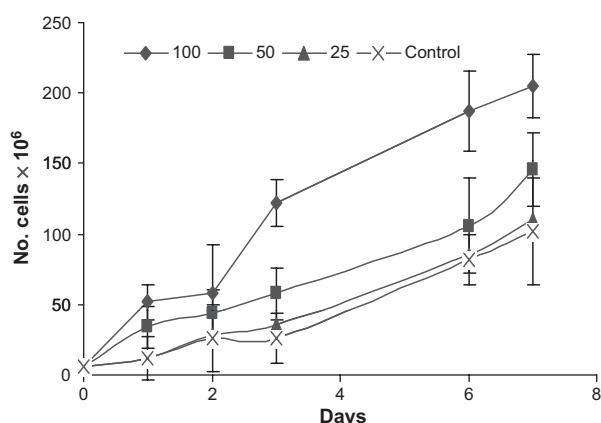


Figure 1 SCA treatment increases the proliferation of HaCaT cells. Increase in the number of HaCaT cells as a function of the days of treatment with different concentrations of SCA (25 , 50 and $100 \mu\text{g mL}^{-1}$). Each point corresponds to the mean value $\pm$ SD from three different experiments. SCA, secretion from the mollusc *Cryptomphalus aspersa*.

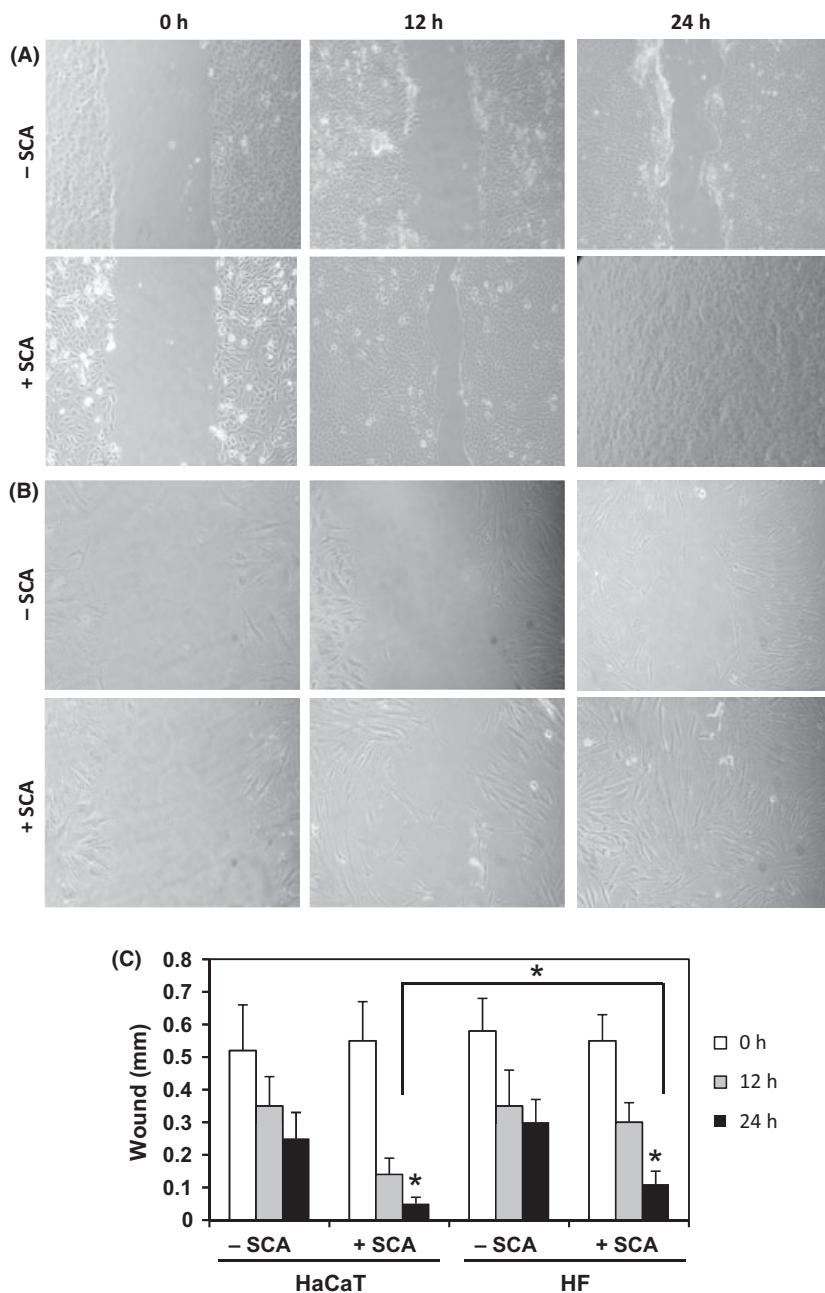


Figure 2 SCA treatment increases the migratory behaviour of HaCaT cells (panels A, C) and human fibroblasts (panels B, C). The motility/migratory behaviour of non-treated (control) or SCA-treated ($100 \mu\text{g mL}^{-1}$, SCA) cells was analysed in an *in vitro* wound model. Confluent cultures were gently scratched with a pipette tip to produce a wound. Photographs of the cultures were taken immediately after the incision (0 h), after 12 h and after 24 h and wounds measured in wide. Pictures are representative of at least 6 experiments. Data of wounds in panel C are given as the mean $\pm$ SD from at least three independent experiments. * $P < 0.05$. SCA, secretion from the mollusc *Cryptomphalus aspersa*.

diminish the effects of either chronological ageing or UV-induced photoageing is increasing dramatically in the last few years.

Among these natural products are fern leaves [18], green tea [19], or retinoids [20]. SCA is a natural secretion from the mollusc *C. aspersa* endowed with regenerative properties in experimental acute radiodermatitis produced in a rat model [21]. In a recent study, Brieva *et al.* [15] demonstrated that SCA also exhibits antioxidant properties and promotes fibroblasts' survival and proliferation and ECM assembly. However, the effects of SCA on keratinocyte biology or other aspects of fibroblast behaviour remained unexplored.

In this report, we show that SCA induces both fibroblast and keratinocyte migration *in vitro*. Throughout the whole repair process, proliferation and migration of different cell types provide formation of the scar. Migration of fibroblasts, which occurs from surrounding unwounded skin towards the provisional matrix of the haemostatic plug, is a crucial step in the wound-healing process [22] that is impaired with ageing [23]. The molecular mechanisms underlying cell migration involve attachment to the ECM, rearrangement of cytoskeleton and subsequent detachment of the cell from the matrix. SCA has already been shown to induce actin reorganization bundling and microfilament alignment in human

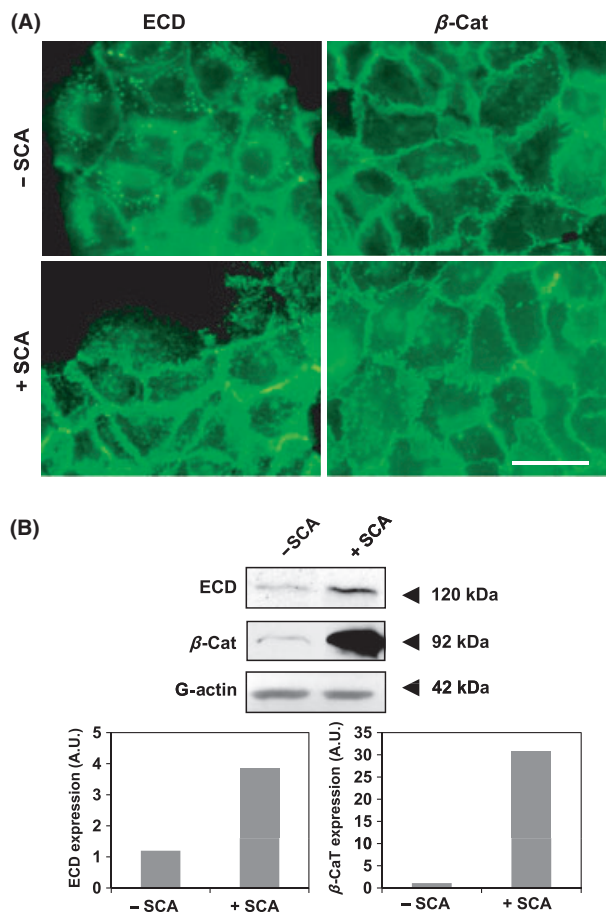


Figure 3 SCA treatment increases the expression of cell-cell adhesion molecules in HaCaT cells. Panel A: Immunofluorescence (IF) staining for E-cadherin (ECD) or β -catenin (β -Cat) in control cells and SCA-treated ($100 \mu\text{g mL}^{-1}$) for 48 h. Preparations were observed under blue excitation light. Note the enhanced ECD and β -catenin expression in SCA-treated cells compared with control cells. Panel B: The SCA-induced increase in cell-cell adhesion molecules expression was also confirmed with Western blot analysis; Densitometric analysis of the bands revealed a 3.2- or 28-fold increase for ECD or β -Cat expression, respectively (lower panel). Scale bar = 20 μ m. SCA, secretion from the mollusc *Cryptomphalus aspersa*.

dermal fibroblasts [15]. Our results show that SCA is able to increase the migration rate of human fibroblasts as well as keratinocytes at 24 h of study, confirming its positive role in the first steps of the wound-healing process. It is of particular interest that SCA also promotes the expression of motility-related molecules, such as β 1 integrins (epidermal and mucosal keratinocytes express α 2 β 1, α 3 β 1 and α 6 β 4 [24], vinculin and FAK), and promotes phosphorylation of FAK, which is a mandatory step in the process of cell migration by participating in the release and reformation of adhesive contacts with the ECM [25]. Our results clearly show that SCA improves the protein production of both molecules in keratinocytes and fibroblasts, thus stimulating the attachment of these cell types to the substrate. Taken together, these findings suggest an important role for SCA in some of the most important events during wound healing that are weakened with age, such as cell-cell and cell-substrate adhesion.

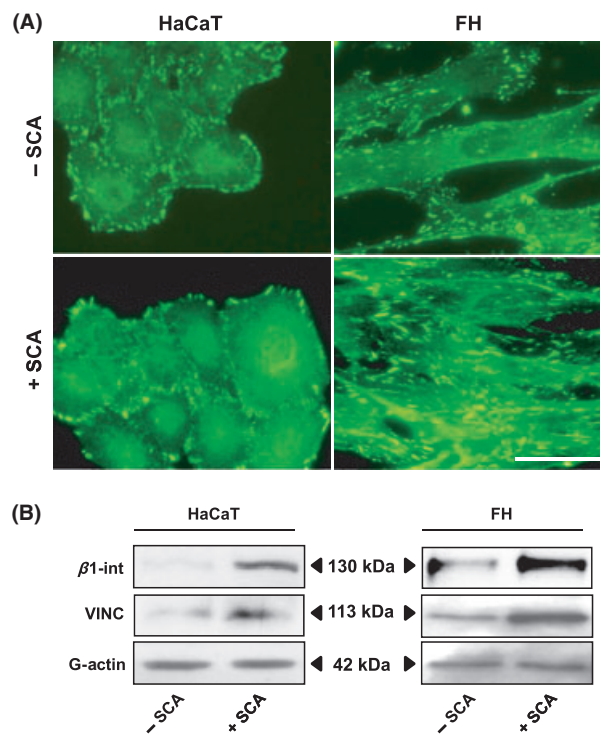


Figure 4 SCA treatment increases the expression of cell-substrate adhesion molecules in HaCaT cells and human fibroblasts. Panel A: Immunofluorescence (IF) staining for vinculin in control cells and SCA-treated ($100 \mu\text{g mL}^{-1}$) for 48 h using HaCaT cells (left panels) or human fibroblasts (right panels). Preparations were observed under blue excitation light. Panel B: The SCA-induced increase in vinculin in HaCaT cells was also confirmed with WB analysis. Expression of β 1-integrin, assayed by Western blot in both cell types, is also increased with SCA treatment (lower panels). Scale bar = 20 μ m. SCA, secretion from the mollusc *Cryptomphalus aspersa*.

Additionally, SCA promotes nuclear appearance of β catenin. Differentiated keratinocytes are characterized by strong intercellular attachment by both desmosomes and adhesion junctions. Adherens junctions are characterized by the presence of ECD, α - and β -catenins, and γ -catenin (plakoglobin) at the membrane [26]. β -Catenin is a multifunctional protein that plays an important role during embryonic development and neoplasia as a mediator of the Wnt signalling pathway [27]. When the Wnt pathway is quiescent, β -catenin participates in adherens junctions [28]. When β -catenin becomes cytoplasmic, it gets phosphorylated and targeted for ubiquitination and degradation. Activation of the Wnt pathway inhibits this phosphorylation, leading to cytosolic stabilization of β -catenin, which consequently translocates to the nucleus where it binds Tcf-Lef transcription factors and regulates transcription [29]. Once in the nucleus, β -catenin interacts with members of the Lef/Tcf family of transcription factors to activate the expression of target genes related to the cell cycle, proliferation and survival. β -catenin translocation to nucleus is associated with several epithelial cancers [30], because it inhibits apoptosis signals and promotes migration and proliferation of epithelial cells [10]. Moreover, in a recent report, it was demonstrated that UVB radiation causes apoptosis, and this process was directly related with cleavage of ECD and its downstream components, including β -catenin [31]. Our results

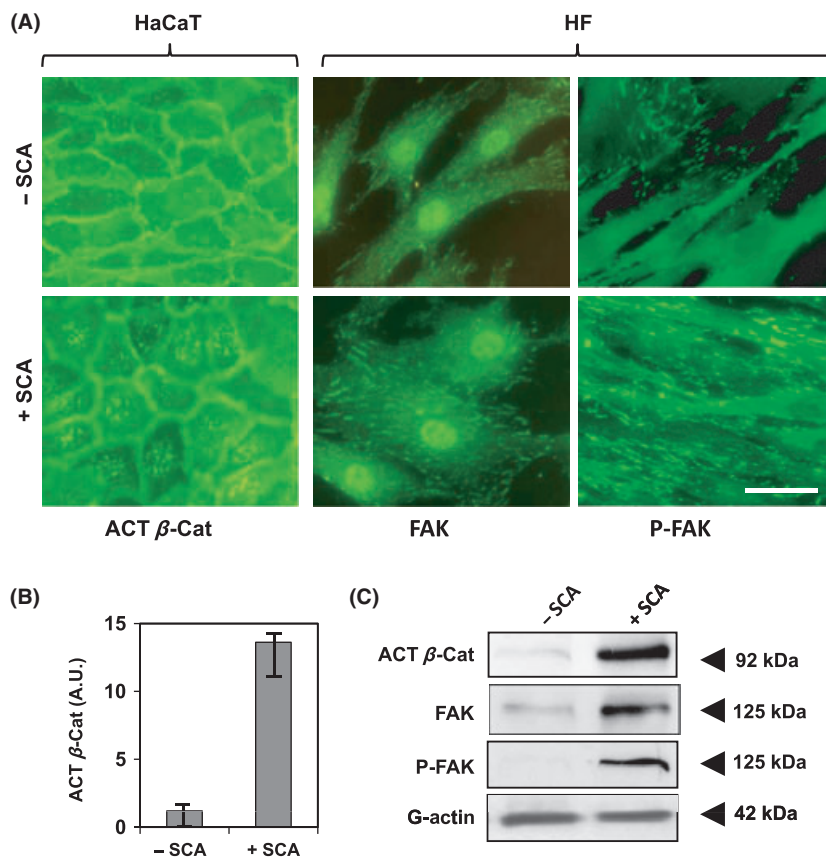


Figure 5 SCA treatment increases the expression of survival molecules in HaCaT cells and human fibroblasts. Panel A shows IF staining for active (nuclear) β -catenin in HaCaT cells and also IF staining for focal adhesion kinase (FAK) and P-FAK in human fibroblasts under control conditions and SCA-treated (100 $\mu\text{g mL}^{-1}$ for 48 h) in both cases. Note the enhanced nuclear expression of active β -catenin in SCA-treated HaCaT cells compared with control cells, and the increased expression of FAK and P-FAK at the membrane zone of human fibroblasts. Panel B: Measurements of fluorescence intensity (arbitrary units, A.U.) of nuclear β -catenin in untreated and SCA-treated cells. Panel C: WB analysis in HaCaT cells confirming both the SCA-increased expression of nuclear β -catenin and the increased expression of FAK and P-FAK under SCA treatment. Scale bar = 20 μm . SCA, secretion from the mollusc *Cryptomphalus aspersa*.

indicate a clear increase in the expression of nuclear β -catenin in cultured keratinocytes after treatment with SCA. This observation clearly points to the ability of SCA in inhibiting keratinocyte apoptosis, although additional studies should be performed.

In addition to its role in regulating cell motility, phosphorylation of FAK also mediates cell survival [32]. At focal adhesions, integrins can activate various protein tyrosine kinases, including FAK. The cytoplasmic tail of integrin β subunits activate FAK, and then, it autophosphorylates Tyr³⁹⁷ creating a binding site for several proteins. These proteins eventually promote signalling pathways that modify the cytoskeleton and activate the extracellular signal-regulated kinase (ERK) family of mitogen-activated protein kinases, leading to cell proliferation [32]. According to Manohar *et al.*, [33] β 1 integrin promotes keratinocyte survival through activation of the ERK cascade, also involving phosphorylation of FAK. Here, we have shown that SCA induces an increase in the expression of FAK

and P-FAK in cultured keratinocytes. Thus, through increased expression of β 1 integrin and phosphorylated FAK, SCA plays a dual role in promoting cytoskeletal reorganization and promoting cell survival.

In summary, our results provide new insights into molecular mechanisms underlying the regenerative properties of SCA. Furthermore, they may suggest dermatologic or cosmetic uses of SCA-based products as to diminish ageing-induced cutaneous manifestations.

Acknowledgements

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