International Journal of
Cosmetic Science

International Journal of Cosmetic Science, 2015, **37**, 41–55 doi: 10.1111/ics.12167

Cryptomphalus aspersa mollusc eggs extract promotes migration and prevents cutaneous ageing in keratinocytes and dermal fibroblasts *in vitro*

J. Espada*, M. Matabuena*, N. Salazar*, S. Lucena*, O. Kourani*, E. Carrasco*, M. Calvo*, C. Rodríguez*, E. Reyes†, S. González‡,§ and A. Juarranz*

*Department of Biology, Faculty of Sciences, Universidad Autónoma de Madrid, Madrid, Spain, †Industrial Farmacéutica Cantabria, Madrid, Spain, ‡Dermatology Service, Memorial Sloan-Kettering Cancer Center, New York, NY, U.S.A. and §Department of Medicine and Medical Specialties, University of Alcalá, Alcalá de Henares, Madrid, Spain

Received 30 July 2014, Accepted 11 September 2014

Keywords: *Cryptomphalus aspersa*, fibroblasts, keratinocytes, senescence, UV protection

Synopsis

BACKGROUND: The search of substances that minimize cutaneous ageing has increased in the last few years. Previous studies have described the regenerative properties of the secretion of the mollusc *Cryptomphalus aspersa* (*C. aspersa*) when applied topically.

OBJECTIVE: We evaluate the *in vitro* effects of a new product derived from the eggs of *C. aspersa*, IFC-CAF, on cell proliferation, migration, distribution of cytoskeletal proteins, production of extracellular components as well as its ability to prevent cutaneous ageing because of intrinsic or extrinsic factors (exposure to UVB) by determination of ageing markers.

METHODS: We have used the human keratinocyte cell line (HaCaT cells), primary dermal fibroblasts (HDF) and senescent dermal fibroblasts (SHDF). The effects of the compound on cell proliferation and on the cell cycle were determined by the MTT colorimetric assay, estimation of total protein and/or trypan blue test and by flow cytometry, respectively. We also studied cell migration using the wound-healing migration assay, whereas ELISA assays, Western Blot and immunofluorescence microscopy were carried out to test the expression of proteins related to cytoskeleton, extracellular matrix and with ageing.

RESULTS: We have found that IFC-CAF does not promote proliferation but induces migration of HaCaT, HDF and SHDF in a time- and dose-dependent manner; a better organization of cytoskeletal proteins (F-actin and vimentin) and promotes the production of extracellular components (fibronectin, collagen 1 and MMPs) and the adhesion to cell-substrate vinculin protein. IFC-CAF also prevents cutaneous ageing. The treatment decreases the expression of the ageing-related markers b-Gal, p53 and p16INK4 in SDDF cells, and improves cell survival after UVB irradiation and nuclear repair in HaCaT cells.

CONCLUSION: IFC-CAF has regenerative properties and protects against ageing factors being, therefore, a potential therapeutic agent for treating or preventing skin ageing.

Correspondence: Salvador González, Department of Medicine and Medical Specialties, University of Alcalá, Alcalá de Henares, Madrid 28871, Spain. Tel.: +34 6102 70045; fax: +34 9143 17805; e-mail: gonzals6@mskcc.org

Résumé

ARRIERE FOND: La recherche de substances qui réduisent le vieillissement cutané a augmenté au cours des dernières années. Des études précédentes ont décrit les propriétés régénératrices de la sécrétion du mollusque *Cryptomphalus aspersa* (*C. aspersa*) lorsqu'il est appliqué par voie topique.

OBJECTIF: Nous évaluons les effets *in vitro* d'un nouveau produit dérivé des oeufs de *C. aspersa*, IFC-CAF, sur la prolifération cellulaire, la migration, la répartition des protéines du cytosquelette, la production de composants extracellulaires ainsi que sa capacité à prévenir le vieillissement cutané dû à des facteurs intrinsèques ou extrinsèques (exposition aux UVB) par détermination de marqueurs du vieillissement.

MÉTHODES: Nous avons utilisé la lignée cellulaire humaine de kératinocytes (cellules HaCaT), les fibroblastes dermiques primaires (HDF) et des fibroblastes dermiques sénescentes (SHDF). Les effets du composé sur la prolifération cellulaire et sur le cycle cellulaire ont été déterminés par le test colorimétrique MTT, l'estimation de protéines totales et / ou un test au bleu trypan et par cytométrie de flux, respectivement. Nous avons également étudié la migration des cellules en utilisant l'essai de migration en tant que modèle de la cicatrisation, alors que les dosages ELISA, Western Blot et la microscopie d'immunofluorescence ont été réalisés pour tester l'expression des protéines du cytosquelette, en relation avec la matrice extracellulaire, ainsi qu'avec le vieillissement.

RÉSULTATS: Nous avons constaté que la IFC-CAF ne favorise pas la prolifération mais induit la migration de HaCaT, HDF et SHDF de manière temps- et dose-dépendante, une meilleure organisation des protéines du cytosquelette (actine F et Vimentin) et favorise la production de composants extracellulaires (fibronectine, collagène 1 et MMP) et l'adhésion à la protéine substrat Vinculine. IFC-CAF empêche également le vieillissement cutané en diminuant l'expression de β-Gal et p53, p16INK4, marqueurs liés au vieillissement dans SHDF, et l'amélioration de la survie des cellules après irradiation UVB et de réparation nucléaire dans les cellules HaCaT.

CONCLUSION: IFC-CAF a des propriétés régénératrices et protège contre les facteurs de vieillissement, étant ainsi un agent thérapeutique potentiel pour traiter ou prévenir le vieillissement de la peau.

Introduction

Skin ageing is a complex biological process influenced by the combination of endogenous (genetics, metabolic processes, hormones, etc.) and exogenous or environmental factors, particularly ultraviolet (UV) light exposure. As a consequence of ageing, progressive loss of physiological integrity appears leading to impaired skin function. UV light is the most important damaging factor that leads to skin photoageing, being a marked increase in the production of reactive oxygen species (ROS), including superoxide anion which can be readily converted into other ROS, and the depletion of endogenous antioxidants (especially at epidermic level), the main mechanisms involved [1, 2]. These events induce photo-oxidative alterations such as lipid peroxidation and DNA damage and a decrease in cell migration and proliferation (both in epidermis and dermis) [3]. Moreover, after skin is injured by an external aggression, the repair machinery is rapidly activated to deal with the damage. These mechanisms include fibroblast proliferation and migration to the wound site to secrete and assemble new extracellular matrix (ECM) components (i.e. collagen, elastin, fibronectin, etc.) that renew skin elasticity and consistency.

In the last few years, the research of natural substances which can improve and stimulate skin regeneration and also exhibit an ageing protective potential, has increased. In this regard, the secretion of the mollusc *Cryptomphalus aspersa* (SCA) has been proved to have antioxidant activity [4–6] and to induce skin regeneration after wound-healing impairment from acute radiodermatitis [7]. Moreover, a recent study demonstrated that SCA stimulates human dermal fibroblast (HDF) proliferation, rearrangement of the actin cytoskeleton and ECM assembly [8]. Other compounds, including resveratrol and curcumin, are under investigation [9, 10].

In this study we have evaluated the regenerative properties of IFC-CAF (the extract of *C. aspersa* eggs) using different *in vitro* approaches. We have found that this extract increases HDF and keratinocyte migration. In addition, we have selected the senescence model of human dermal fibroblasts (SHDF) induced by sequential passages to detect the potential protective role of IFC-CAF in ageing. We have also selected the UV-induced premature senescence model in HaCaT to investigate the protection of IFC-CAF against this external ageing factor and the associated molecular mechanisms. We have found that IFC-CAF considerably prevents senescence-like phenotypes in the UV challenge model, including alterations of morphology, cell cycle, β-Gal staining, DNA damage, as well as related molecules expression such as p53 and p16. This finding presents IFC-CAF as an attractive product (agent/ingredient) with potential to retard ageing and attenuate age-related phenotype in human cells.

Materials and methods

Reagents

IFC-CAF was prepared according to US patent US 5538740. Briefly, gastropod spawn were rinsed with distilled water at a very low pressure, immersed in saline solution and kept between 2°C and 8°C. Then, the saline solution was filtered through a mesh with a diameter of less than 3 mm to obtain intact snail spawn remain. Spawn were lysed and homogenised by means of a Silverson mill and filtered again. The liquid obtained was used in this work, and it was obtained at a concentration of protein of 27 mg mL^{-1}, determined by the BCA protein assay (see below). Other drugs and reagents used in this study were as follows: 3-[4,5-dimethyl-2-thiazolyl]-2,5-diphenyl-2-tetrazolium bromide (MTT) (Sigma-Aldrich, St. Louis, MO, U.S.A.); Senescence β-Galactosidase Staining Kit (Cell Signaling, Danvers, MA, U.S.A.); propidium iodide (PI) and neutral red (NR), both from Merck (Darmstadt, Germany); and paraformaldehyde (Panreac, Barcelona, Spain). For cell cultures, plastic materials and mediums were obtained from Costar (Corning, NY, U.S.A.) and from Gibco (Paisley, Scotland, U.K.), respectively. For immunodetection of the evaluated proteins, the primary antibodies used were rabbit polyclonal anti-fibronectin, vimentin, Ki67 (Abcam, Cambridge, U.K.), collagen 1 (Santa Cruz Biotechnologies, Dallas, TX, U.S.A.), p53, p16, monoclonal anti-β-actin, vinculin (Sigma-Aldrich), Keratin (Biomeda, Foster City, CA. U.S.A.), acetylated histone H3 (Lys 9) (AcK9H3, Upstate, Millipore, Bedford, MA, U.S.A.), trimethylated histone H3 (Lys4) (3mK4H3, Abcam), fibrillarin, and anti-phospho-H2AX (Ser 139; Cell Signaling). The secondary antibodies used were HRP monoclonal antibody anti-mouse IgG and HRP monoclonal antibody anti-rabbit IgG (Amersham Pharmacia Biotech, Little Chalfont, U.K.), monoclonal antibodies anti-mouse IgG and anti-rabbit IgG FITC or TRITC-labelled (Sigma-Aldrich). All other reagents were from commercial suppliers and of standard biochemical quality.

Cell cultures and treatments

The cells included in the study were HaCaT cells, an immortalized human keratinocyte cell line, human dermal fibroblasts (HDF) and senescent dermal fibroblasts (SHDF) obtained by repeated passages of HDF. The cells were considered to be young with less than 20 population doublings (PD), pre-senescent between 21 and 29 PD and senescent cultures at PD 30 or later and having more than 50% β-Gal positive cells (assayed using the Senescence β-Galactosidase Staining Kit). The cells were grown in F-25 flasks, 35-mm culture dishes or 22-mm square glass coverslips placed into dishes, using Dulbecco's modified Eagle's medium (DMEM) containing 10% (v/v) foetal calf serum (FCS), 50 U mL^{-1} penicillin, 50 mg mL^{-1} streptomycin and 1% (v/v) 0.2 M L-glutamine. Cells were incubated at 37°C in an atmosphere containing 5% CO_2.

For treatments, IFC-CAF was partially dissolved in fully supplemented DMEM under intense agitation for at least 30 min at 37°C. Samples were further filtered through 0.22-μm syringe filters giving a final concentration of 0.77–0.82 mg mL^{-1}. For routine experimental treatments, cells were grown in DMEM-dissolved IFC-CAF for variable times (from 15 h to several days) depending on the study and replacing the culture medium every 2 days to maintain the IFC-CAF concentration relatively constant over time. For UV-induced premature senescence experiments, HaCaT cells were treated with IFC-CAF for 15 h and immediately irradiated with 250 nm UV light at 25 (moderate condition) or 50 (severe condition) J m^{-2}. Cells were collected and processed for different assays 5 h after irradiation.

Cell viability/proliferation assay

Cell viability/proliferation was measured by the MTT assay [11]. Cells cultured on 12- or 24-well Petri dishes were treated with different concentrations of IFC-CAF (5, 25, 50 and 100 μg mL^{-1}) during several time points (from 1 to 7 days). Following IFC-CAF treatment, MTT solution was added to each well at a concentration of 0.5 ng mL^{-1}, and plates were incubated at 37°C for 3 h. The resulting formazan crystals were dissolved by the addition of DMSO,

and absorbance was measured at 560 nm. The viability was also by microscopy evaluation with a trypan blue exclusion test using a Neubauer chamber counter. In addition, cell proliferation was estimated by measuring the amount of protein in the cultures treated or not with *C. aspersa.* After treatment, cells were treated with RIPA buffer (150 mM NaCl, 1% Triton X-100, 0.05% deoxycholate, 0.1% SDS, 1% Nonidet-40, 50 mM Tris, pH 8) containing Phosphatase Cocktail 2 and Protease Inhibitor Cocktail (Sigma, St Louis, MO, U.S.A.). The estimation of protein concentration in the cultures was performed using the BCA Protein Assay Reagent (Pierce, Rockford, IL, U.S.A.).

Cell cycle analysis

Cells treated with IFC-CAF were treated for the indicated time. Afterwards, cells were trypsinized, resuspended in 0.5 ml of PBS and fixed with 75% ethanol at 4°C overnight. After RNase A digestion (0.1 mg of RNase A at 37 8°C for 0.5 h), the cells were stained with 50 mg mL^{-1} PI for 30 min before analysis with flow cytometer (Becton-Dickinson FACScalibur, San Jose, CA, U.S.A.) using CELL Quest software (at least 10 000 events were collected).

Neutral red staining

To detect morphological changes after treatments, cells grown on coverslips were fixed for 5 min in cold methanol and stained for 30 s with neutral red (NR) at 0.05 mg mL^{-1} in distilled water, washed with distilled water and air-dried. Preparations were mounted in DePeX and observed under bright-field microscopy.

TUNEL assay

To detect DNA fragmentation, the terminal deoxynucleotidyl transferase-mediated dUTP nick end-labelling (TUNEL) assay was performed at different times after treatments. Cells grown on coverslips were fixed in 3% paraformaldehyde for 15 min at 4°C, washed three times in PBS, permeabilized with 0.1% (v/v) Triton X-100 in PBS for 2 min and incubated with TUNEL reaction mixture (Roche, Indianapolis, IN) for 1 h at 37°C. Cells were washed with PBS, mounted in ProLong Gold (Molecular Probes, Carlsbad, CA, U.S.A.) and observed under fluorescence microscopy (blue light excitation).

Migration assay

In vitro migration by scratch wound assay was performed as previously described [8]. Briefly, HaCaT and HDF cells were seeded in P6-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 IFC-CAF was applied to 3 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 camera (Olympus, Tokyo, Japan) connected to the microscope. Quantification of migrated cells was performed by measuring the healed distance between the borders of the scratches. Data are given as means ± SD of three independent experiments. Statistical significance was evaluated using the Student′s *t*-test, and differences were considered to be significant at a value of $P < 0.05$ (*).

Immunological methods

Exponential cell cultures were treated with different concentrations of IFC-CAF for different time points. For general immunodetection, cells were fixed in 3% paraformaldehyde for 15 min, washed in PBS, permeabilized with 1% Triton X-100 in PBS for 10 min and incubated for 1 h at 37°C with the corresponding primary antibodies. Afterwards, coverslips were washed with PBS, incubated with the secondary antibodies and finally washed with PBS. The preparations were mounted in Prolong reagent containing DAPI for nuclear staining (Invitrogen, Carlsbad, CA, U.S.A.).

For Western blot analysis, treated monolayers were washed and then lysed with RIPA buffer 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 μg 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 destaining, they were blocked with 5% skimmed milk in Tris-buffered saline (Tris–HCl 10 mM pH 7.6, 0.9% NaCl, 0.05% Tween 20). The membranes were incubated with the following specific antibodies: G-actin (Sigma), diluted 1 : 500 in blocking buffer overnight at 4°C and HRP secondary monoclonal antibody anti-mouse Ig G was diluted 1 : 1000 and used to incubate the membranes 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 (GE Healthcare, Hertfordshire U.K.).

For ELISA (enzyme-linked immunosorbent assay), the conditioned medium below each treated culture was collected, centrifuged at 1000 *g* for 10 min and analysed by the corresponding ELISA kits (Abcam) to determine the secreted human proteins: fibronectin (ab108847), MMP-2 (ab100606) and MMP-9 (ab100610) according to the manufacturer's recommendations. The absorbance was measured at 490 nm.

β-Gal activity analysis

β-Gal activity was determined using a Senescence Detection Kit. SHDF were incubated with different concentrations of IFC-CAF (100 ng mL^{-1} to 100 μg mL^{-1}) for variable times (from 1 to 6 days). Afterwards, cells were washed with PBS and subjected to the senescent assay. As positive controls, cells were incubated with 150 μM H_2O_2 for 1 h, H_2O_2. Then, H2O2 was removed from the culture, and the cells were incubated in fresh culture medium containing 10% FCS for 1–6 days. Senescent cells were identified as blue-stained cells by standard light microscopy. A total of 600 cells were counted in three random fields on a culture plate to determine the percentage of β-Gal positive cells.

Cell adhesion and spreading assay

The assay was based on the previously published [12]. HDF and SHDF were cultured for 1 week in the presence and in the absence of 100 μM IFC-CAF. At the end of the treatment, cells were serum-starved for 24 h before detaching with 0.25% trypsin. Trypsin was blocked with DMEM complete medium, centrifuged and resuspended in 2 mL of fresh medium. About 10^5 cells were seeded in P12 plates

containing complete DMEM medium. Cells were observed and photographed at different time points for a period 5 h under the phase-contrast microscope. At the end of the experiment, cells were fixed with methanol–acetic acid (3 : 1) solution and then stained with 0.5% crystal violet in 2% EtOH. After solubilization with 1 mL per well with 2% SDS in PBS, density of cells adhering to dish was quantified at 580 nm with a spectrophotometer.

Microscopic observations and statistical analysis

Microscopic observations and photographs were performed with an Olympus photomicroscope BX61 (Tokyo, Japan) 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). Data are expressed as mean ± SD. The statistical significance was determined using Student's *t*-test and analysis of variance (ANOVA). $P < 0.05$ was considered statistically significant.

Results

IFC-CAF modulates cell turnover in HaCaT, HDF and SHDF cultures

First, we tested whether IFC-CAF affected the proliferation of HaCaT, HDF and SHDF cells. The cultures were incubated with different concentrations of IFC-CAF for variable times and evaluated by different tests. Using the MTT assay, we found a significant decrease in cell proliferation when the compound was used at a high concentration (500 µg mL^{-1}) for 2 days of treatment in HaCaT, HDF and SHDF cells ($P < 0.05$), whereas the remaining concentrations evaluated did not affect significantly cell proliferation compared to control cells (Fig. 1A). We also evaluated cell proliferation by measuring the total protein amount in the cultures after treatment up to 7 days with IFC-CAF at a selected concentration of 10 µg mL^{-1}, confirming that up to 6 days treatment with the product did not significantly affect cell proliferation in any of

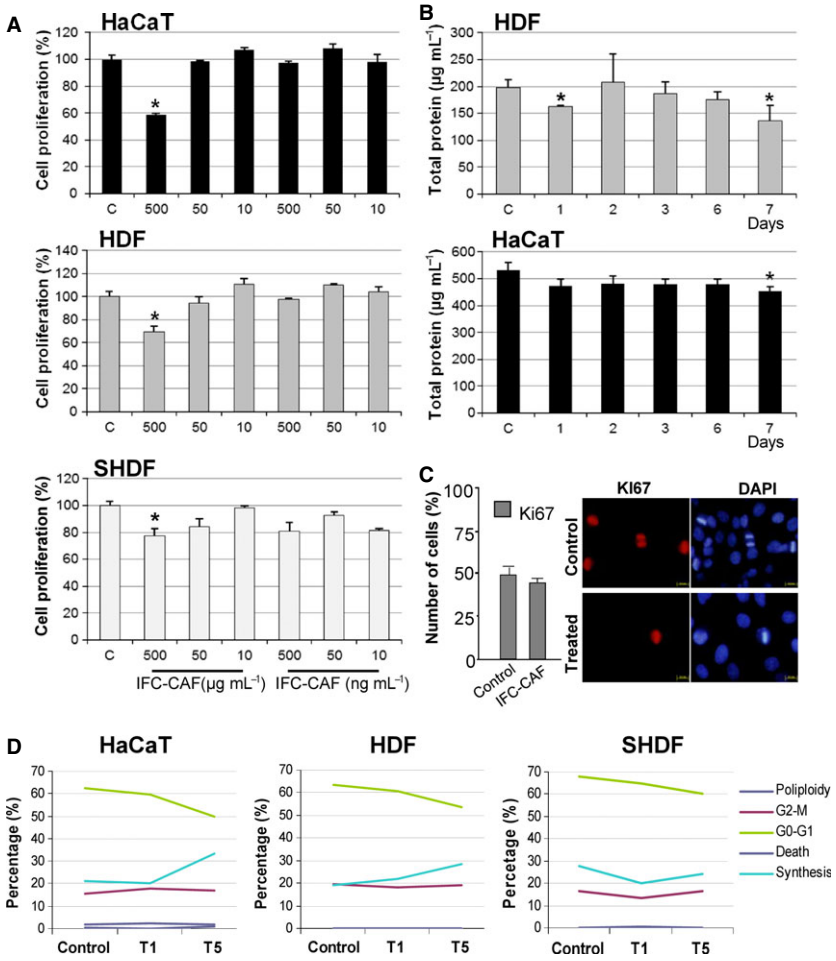

Figure 1 (A) Cell proliferation of HaCaT cells, HDF and SHDF measured by the MTT assay after treatment with different concentrations of IFC-CAF (500 µg mL^{-1} to 10 ng mL^{-1}) for 2 days. (B) Cell proliferation of HaCaT cells and HDF estimated by total protein in the cultures after treatment with 10 µg mL^{-1} of IFC-CAF for variable times. (C) Number of HaCaT Ki67-positive cells in control and treated cells. (D) Distribution of HaCaT, HDF and SHDF cells in the cellular cycle determined by flow cytometry, controls and treated cells with 10 µg mL^{-1} of IFC-CAF. * P<0.05 compared to Control.

the cell types, HaCaT, HDF (Fig. 1B) and SHDF (data not shown); however, a significant decrease in cell proliferation was detected when the treatment was administered for 7 days ($P < 0.05$). In HaCaT cultures, the estimation of the number of Ki67 positive cells indicated that such concentrations of IFC-CAF did not induce an increase in the number of labelled cells (Fig. 1C). Similar results were obtained with HDF and SHDF. All these results indicate that the compound did not induce proliferation in these cells under our experimental conditions. From the above results, we decided to select the concentration of 10 μg mL^{-1} and a 5 days of treatment for most of the following experiments because no significant changes in cell proliferation were observed and allowed evaluating the long-term effect of IFC-CAF.

As no substantial alterations on cell proliferation were observed after IFC-CAF treatment, we decided to evaluate the effects of the product on the cell cycle of HaCaT, HDF and SHDF (Fig. 1D). Treatments for 1 and 5 days at a concentration of 10 μg mL^{-1} did alter the distribution of HaCaT cells and HDF in the different phases of the cell cycle. We found a decrease in the number of cells in G1 phase and an increase of the number of cells in S and G2-M phases, particularly noticeable after 5 days of treatment. The distribution of the cells in the cell cycle was scarcely modified in SHDF after 1 day of the treatment, and after 5 days.

IFC-CAF affects cellular morphology but not nuclear stability

We next tested whether IFC-CAF affected cell morphology and/or nuclear stability (Fig. 2). Morphological observations after NR staining indicated that HaCaT cells treated for 5 days with 10 μg mL^{-1} with IFC-CAF presented a more evident polyhedral morphology and a more basophile cytoplasm compared to that of control cells (Fig. 2A). In addition, HDF cultures showed a more organized morphology; the cells appeared growing with the main axis in parallel. The effects on SHDF were similar to that observed

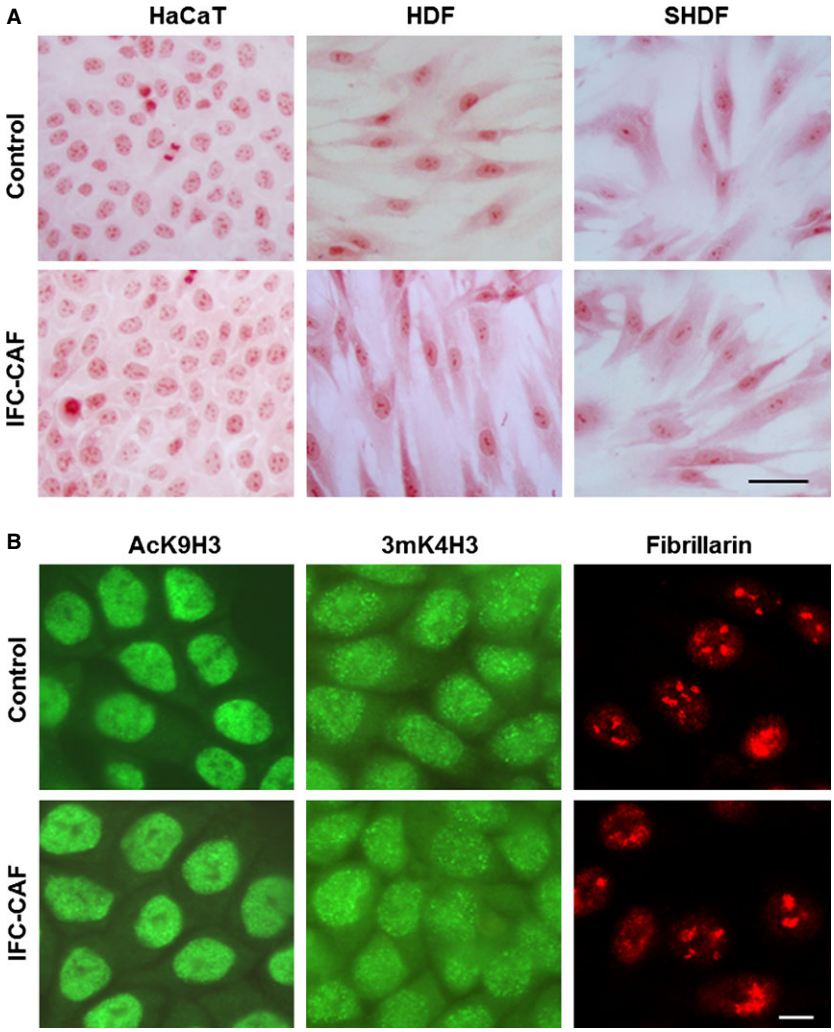

Figure 2 (A) Cellular morphology after IFC-CAF treatment in HaCaT cells, HDF and SHDF at 10 μg mL^{-1} for 5 days. Scale bar: 20 μm. (B) Immunolocalization of euchromatin, heterochromatin and nucleolar compartment markers: AcK9H3, 3mK9H3 and fibrillarin in HaCaT cells, untreated (Control) and treated with IFC-CAF at 50 μg mL^{-1} for 24 h of treatment. The results are representative of two independent experiments. Scale bar: 10 μm.

in HDF; whereas SHDF *in vitro* are much larger with round and flat appearance compared to that of HDF, treated SHDF presented a better organized morphology. As at the 10 μg mL^{-1} concentration we did not observe evident nuclear changes after NR staining, we decided to evaluate the treatment for 24 h with a higher concentration (500 μg mL^{-1}) selecting the HaCaT cells to test it (Fig. 2B). We did not detect alterations neither in the distribution pattern of euchromatin, determined by acetyl-Histone H3 and trimethyl-Histone H3 labelling, nor in the nucleolar architecture, using an antibody for fibrillarin. Cells presented expression and distribution patterns were quite similar in treated and untreated (control) HaCaT cells (Fig. 2B). These results indicate that the compound does not affect nuclear stability.

IFC-CAF treatment stimulates migration of HaCaT and HDF

Next, we evaluated whether IFC-CAF treatment favoured migration of keratinocytes and HDF, using a classical migration assay. Confluent cultures of HaCaT, HDF and SHDF cells were gently scratched with a pipette tip and treated with variable concentrations of IFC-CAF for 72 h. Photographs of the scratch area were taken at times 0, 12 and 24 h (Fig. 3A). IFC-CAF promotes

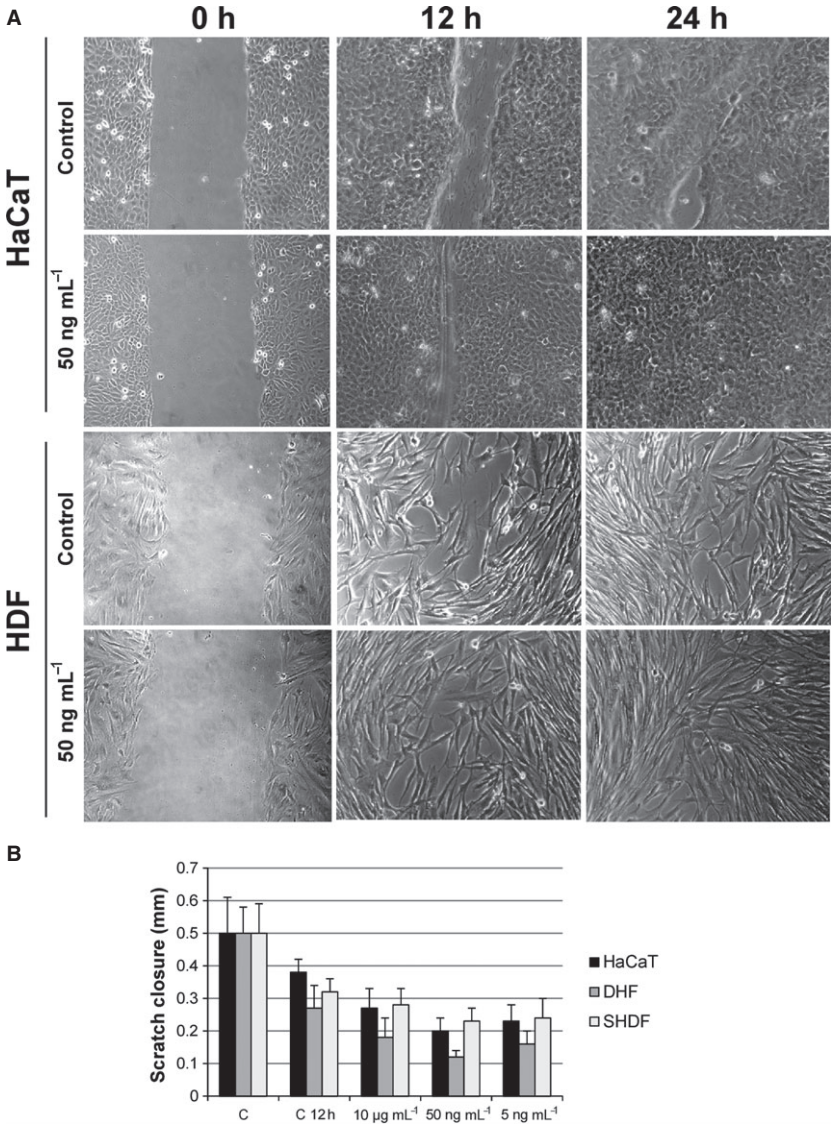

Figure 3 IFC-CAF treatment increases the migratory behaviour of HaCaT cells and HDF (panels A). The motility/migratory behaviour of non-treated (control) or IFC-CAF-treated (50 ng mL^{-1}) cells were analysed in an *in vitro* wound model. Confluent cultures were gently scratched with a pipette tip to produce a wound. Photographs of the cultures were taken immediately after the incision (0 h), 12 h and after 24 h, and wounds measured in wide. Pictures are representative of at least 3 experiments. (B) Data of wounds, including SHDF, are given as the mean ± SD from at least three independent experiments. Significant differences were seen between controls and cells treated with 50 or 5 ng mL-1 IFC-CAF. *$P < 0.05$.

occupation of the space between both limits of the scratch compared to untreated control cells. Treatments with 10 μg mL^{-1} and 50 ng mL^{-1} of HaCaT were the best concentrations for scratch closure in this cell type in relation with untreated controls ($P < 0.01$). In the same way, scratch closure of HDF was faster under treatment with all the concentrations of IFC-CAF tested comparing to DHF controls ($P < 0.01$); 10 μg mL^{-1} of IFC-CAF was the best concentration for HDF scratch closure ($P < 0.01$). Finally, it should be noted that HDF closed earlier than HaCaT cells at any of the concentrations evaluated; 24 h after IFC-CAF addition, the wound was completely occupied with HDF, whereas there was still an open space in HaCaT cells for the same treatment conditions (Fig. 3B).

IFC-CAF treatment promotes the production of extracellular matrix components

The potential modifications in the production of fibronectin (FN) and collagen 1 (Col1) were evaluated by immunofluorescence (Fig. 4A). The production of FN was also evaluated by ELISA (Fig. 4B). The observations with the microscope revealed that treated HaCaT and HDF cells showed a higher expression of FN as well as a higher organization with respect to controls; as we observed after NR staining, HDF tended to be organized in parallel with a high expression of this protein (situated at the extracellular level). The expression of FN seems to be related with the concentration, being higher with a concentration of 10 μg mL^{-1}. The results obtained by immunofluorescence were corroborated by ELISA. Similar results were obtained on Col1 production as it can be observed in Fig. 4A, and an increase in the red fluorescence (because of Col1 production) could be observed in the treated cells compared to that observed in untreated cells. No relevant differences were observed between the IFC-CAF concentrations tested.

In keeping with the effects of IFC-CAF on cell-matrix components, and knowing the importance of metalloproteinases (MMPs), the enzymes that break down collagen [13], in skin remodelling and ageing, we have also evaluated the variations in the

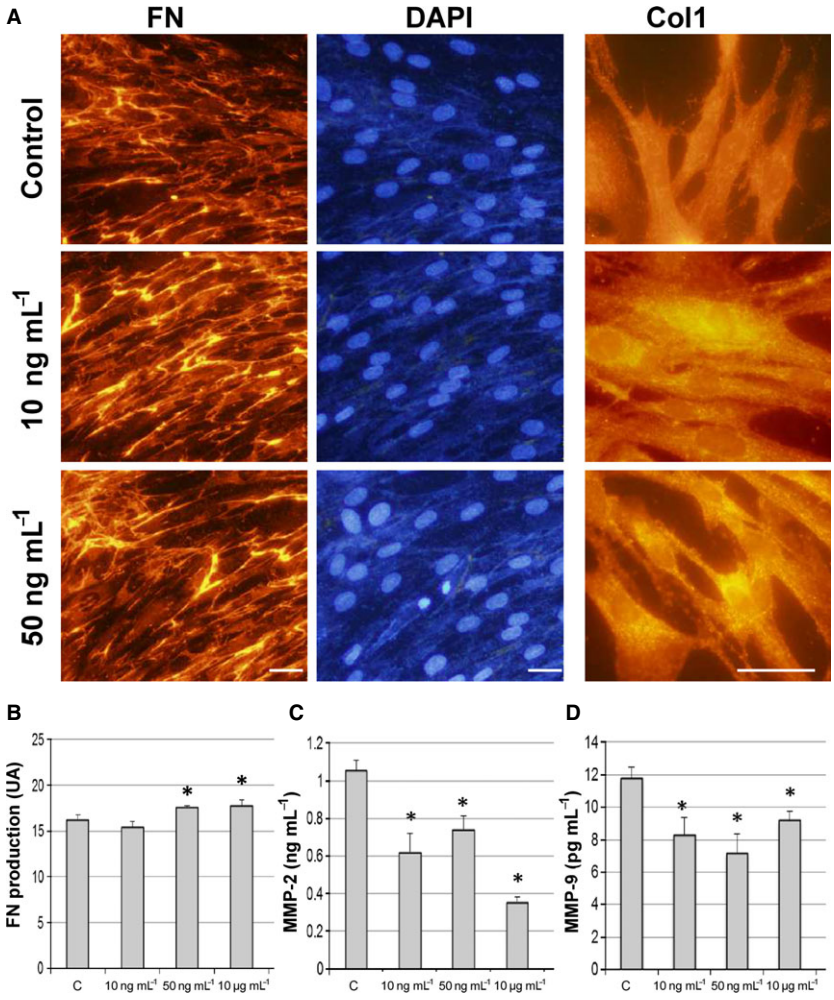

Figure 4 (A) Fibronectin (FN) and Col1 expression determined by immunofluorescence in HDF untreated and treated with IFC-CAF. FN appears fluorescing in red and nuclei counterstained with DAPI in blue under green and UV exciting light. Scale bar: 20 μm. (B) Production of FN; (C) MMP-2 (ng mL^{-1}) in HDF; and (D) MMP-9 (pg mL^{-1}) in HaCaT cells determined by ELISA. It can be observed a significant increase in FN production and decrease of both MMPs in the presence of IFC-CAF. * $P<0.05$ compared to Control.

production of MMP-2 in HDF and MMP-9 in HaCaT induced by the treatment with IFC-CAF. As it can be observed in Fig. 4 (C and D, respectively), the treatment induced a decrease in the production of both MMPs. The decrease was statistically significant ($P < 0.05$) compared to control cells for MMP-2, but no statistical differences were observed between the concentrations of 10 μg mL^{-1} and 50 ng mL^{-1}. Therefore, IFC-CAF decreases the production of MMP-2 even at low concentrations. In the case of MMP-9, there were also significant decreases in relation to controls ($P < 0.05$), but no statistical differences were observed among the concentrations used.

IFC-CAF treatment affects the cytoskeleton organization

The cytoskeleton is a key factor involved in both cell shape and elasticity in the division process. Thus, the effects of IFC-CAF on the expression and organization of vimentin, which configures the intermediate filaments in HDF and SHDF, the expression and organization of stress fibres (F-actin), and the cell-matrix adhesion marker vinculin were studied, in the three cell types: HaCaT, HDF and SHDF.

A characteristic of senescent skin fibroblasts is that they produce high amounts of vimentin relative to actin and tubulin, which are down-expressed, and also exhibit decreased adhesion to substrate [14]. Figure 5 shows the results obtained in terms of the distribution and expression of vimentin determined by immunofluorescence or Western blot, respectively. HDF had a well-defined network of fine filaments of vimentin, whereas this protein appeared more punctate and forming some thick filaments in the cell ends of SHDF. Treatment with IFC-CAF partially prevented this effect, and the treated SHDF cells showed, by immunofluorescence, a similar expression and distribution of vimentin compared to HDF (Fig. 5A). These results were confirmed by Western blot; whereas no differences were seen for overall vimentin production by treatment with IFC-CAF in normal HDF, this protein's levels were diminished in the case of SHDF, which had a higher amount of vimentin when untreated (Fig. 5B).

The distribution of actin filaments in HDF and SHDF is shown in Fig. 6. HDF had a well-defined network of F-actin, which is formed by long and thin filaments of this protein and numerous and prominent focal contacts (determined by the expression of vinculin). Stress fibres of SHDF apparently showed greater thickness and were shorter than those of the HDF, and focal contacts appeared thinner than those of non-senescent cells. IFC-CAF treatment resulted in changes in the distribution of actin filaments and focal contacts of senescent cells, showing a similar morphology to that of controls; the treatment promotes the recruitment of vinculin to focal adhesions, which is correlated with an increased vinculin expression.

In the same way, the expression of actin and vinculin was analysed in untreated and IFC–CAF-treated HaCaT keratinocytes (Fig. 6). As shown, the actin filaments are located in both treated and untreated cells in the subcortical region, without significant differences among them. There are a greater number of focal contacts in treated cells, which are particularly conspicuous at the level of cells on the colony edges.

Effects on β-Gal activity and expression of p53 and p16

We have also evaluated the ability of IFC-CAF to prevent the effects caused by the senescence process induced by intrinsic factors in SHDF. The process is characterized by an increase of β-Gal positive cells and by changes in the expression of known senescence markers such as p16 and p53 [15]. Different concentrations of the product (100 ng mL^{-1}, 1 μg mL^{-1} and 10 μg mL^{-1}) and different incubation times (1–7 days) were analysed (Fig. 7). The results showed that IFC-CAF treatment (100 ng mL^{-1}) did not cause substantial changes from controls in the number of β-Gal positive cells and in the expression of p16 and p53 (data not shown). However, we found differences between treatments at 1 and 10 μg mL^{-1}, being apparently higher the changes with the concentration of 10 μg mL^{-1}, so only these results are shown in Fig. 7.

In the morphological studies (Fig. 7A), β-Gal positive senescent cells showed a blue colour, being the percentage of these cells in cultures of HDF very low. This percentage increased significantly in senescent cells (passage 30), but decreased after IFC-CAF treatment (Fig. 7B). Likewise, there was a change in the location of p16 in HDF compared to SHDF, being predominantly cytoplasmic in the first case, whereas in the second one was both cytoplasmic and nuclear (Fig. 7A). IFC-CAF treatment partially prevented the localization of p16 in SHDF, which showed a similar expression to that observed in HDF.

Another important marker of senescence is p53, this protein is overexpressed in SHDF cells, whereas IFC-CAF treatment appeared to reduce its expression (Fig. 7C, top). The reduced expression is directly related with treatment time, being lower after 7 days of treatment. No striking changes were observed in the expression of p53 in HDF or HaCaT cells, relative to controls (Fig. 7C, bottom).

Cell adhesion

In addition, we have evaluated the capacity of adhesion of HDF and SHDF after treatment with IFC-CAF. The adhesion and spreading on plate was impaired in SHDF, and 4 h after seeding, cells were not well attached to the substrate compared to HDF. Treatment with IFC-CAF significantly improved the adhesion ability of HDF, but especially of SHDF as shown by morphological evaluation and by the crystal violet assay (Fig. 8).

Prevention of UV-induced premature senescence by IFC-CAF

Finally, we also selected the UVB-induced premature senescence model in HaCaT cells to investigate the protective effect of IFC-CAF against this environmental extrinsic ageing factor. To induce damage in HaCaT cells (evaluated by TUNEL assay, Fig. 9B), two experimental UVB radiation were established: 25 and 50 J m^{-2} UVB exposure was performed immediately after 24 h of treatment with 50 μg mL^{-1} IFC-CAF, and the evaluation was carried out 5 h after irradiation. DNA damage was determined by immunofluorescence for γ-H2AX, which is phosphorylated in response to double strand DNA breaks. We also evaluated the changes in the expression of sirtuin 1 (Sirt1), a molecule related with senescence. The results obtained are shown in Fig. 9. As it can be seen, IFC-CAF protects against the damage induced by UVB; whereas an increase in the positivity to γ-H2AX in UVB-irradiated cells was detected, cells treated with IFC-CAF prior to UVB irradiation showed a lower expression of this protein, similar to that of controls (Fig. 9A). A decrease in the expression of Sirt1 was observed in both unirradiated cells treated with IFC-CAF and cells subjected to UVB after treatment with IFC-CAF. Immunofluorescence results were obtained by counting the number of positive cells for the evaluated markers (Fig. 9B). First, treatment with IFC-CAF did not induce an increase in the number of TUNEL-positive cells (apoptotic cells), confirming

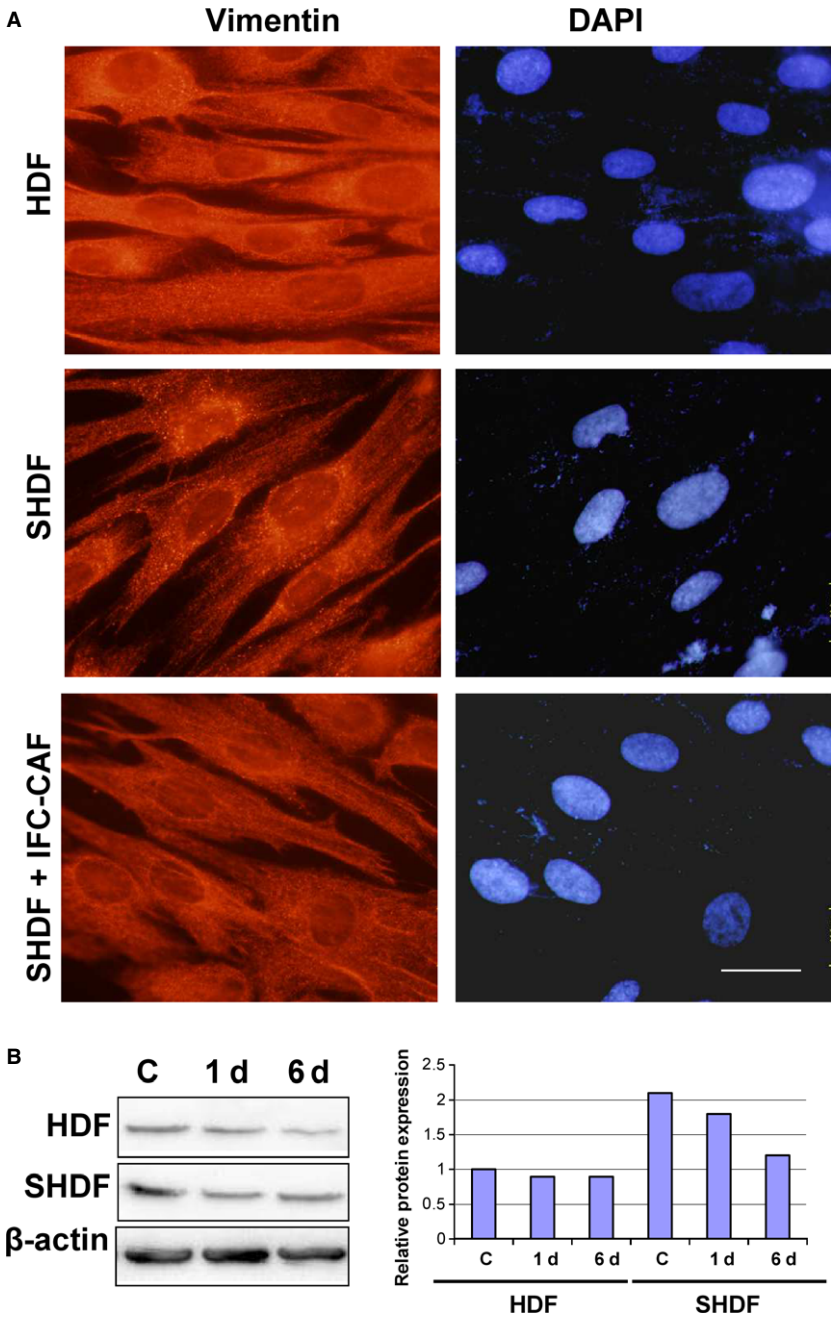

Figure 5 (A) Vimentin expression determined by indirect immunofluorescence on HDF and SHDF cells, treated with 10 μg mL^{-1} of IFC-CAF for 6 days. Vimentin appears in red, and nuclei in blue by DAPI counterstaining, under light green or ultraviolet excitation, respectively. Scale bar: 20 μm. (B) Expression of vimentin by Western blot showing that IFC-CAF treatment reduces the expression of the protein in SHDF.

the results obtained in the toxicity assays showed in Figs 1 and 2. Treatment with IFC-CAF alone did not induce significant increase in the number of positive cells for γ-H2AX. The protective effect of IFC-CAF could be observed when cells, after treatment, were exposed to UVB radiation as the number of positive cells for γ-H2AX decreased to reach almost control values. The number of positive cells for Sirt1, however, was maintained in all the evaluated conditions. Western blot analysis revealed a decrease in the expression of γ-H2AX in cells previously treated with IFC-CAF and then exposed to UVB radiation confirming their protective effects

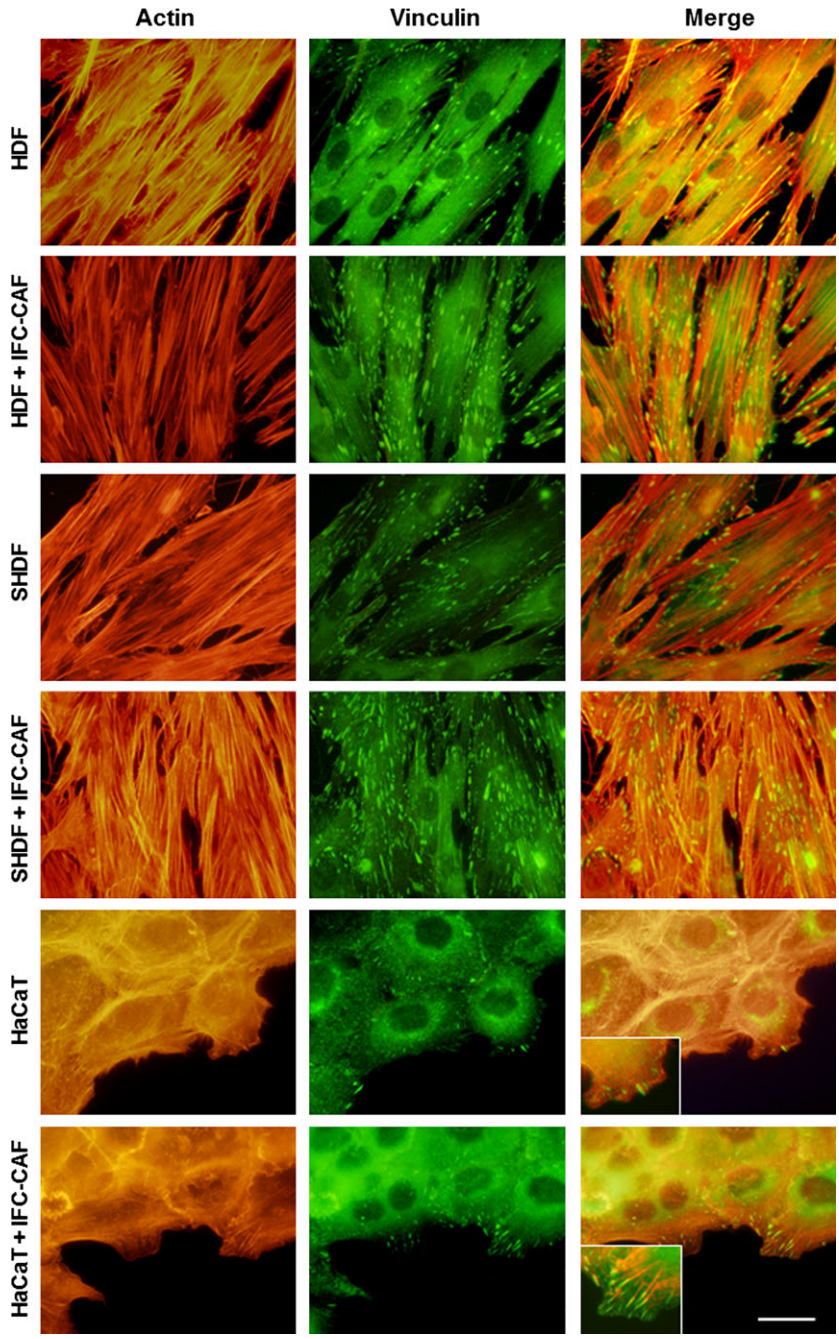

Figure 6 Effects of IFC-CAF treatment with 10 μg mL^{-1} for 6 days on the morphology and distribution of stress fibres (left panel) and focal contacts (middle panel) of HDF, SHDF and HaCaT cells. Stress fibres appear in red under green excitation light, determined by specific TRITC-phalloidin staining. The focal contacts were determined by indirect immunofluorescence for vinculin. The right panel shows the merge of the images of the middle and left panels. The inserts in the right panels show the merge of the images of TRITC-actin F and vinculin in regions of focal contacts of HaCaT cells. Scale bar: 20 μm.

(Fig. 9C). However, although under such irradiation conditions IFC-CAF do not increase the number of cells that expresses SIRT-1, it does affect the level of expression of this protein. There was a decrease in the expression of Sirt1 in all the evaluated conditions, even when the cells were only treated with IFC-CAF.

Discussion

Skin ageing is a complex sequence of events that reflects the changes occurring in both intrinsic and extrinsic ageing (e.g. UV exposure). The process is thought to be driven by an increased *in*

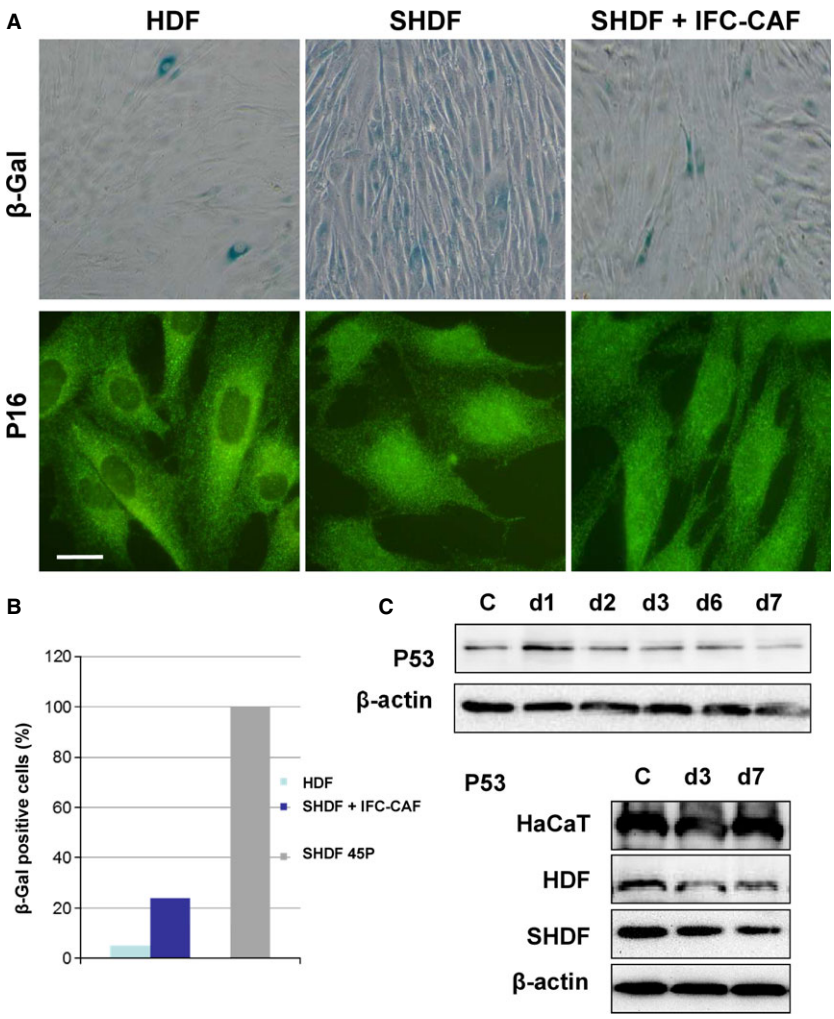

Figure 7 (A) and (B) Effects of IFC-CAF treatment in the number of β-Gal positive cells; SHDF cultures (passage 30) have a greater number of blue cells (β-Gal positive) than non-senescent HDF; IFC-CAF treatment reduced the number of positive cells for this marker. As a positive control, 45 passes of SHDF were used. p16 expression in IFC-CAF-treated HDF and SHDF. (C) Western blot for p53 in HDF, SHDF and HaCaT, controls and treated samples for different times at 10 μg mL^{-1} IFC-CAF.

situ production of ROS, which impairs remodelling capacity of the skin, and reduced cell migration [1, 2]. There is also good evidence that skin ageing is associated with depletion of naturally occurring antioxidants that serve as a defence mechanism against free radical damage [16]. Therefore, natural products that can diminish the effects of ageing are under investigation, such as fern leaves [17], green tea [18], retinoids [19], resveratrol [9] and also SCA, which has been proved to have regenerative properties as well as antioxidant activity [4–7]. In this work, the product IFC-CAF obtained from the mollusc *C. aspersa* has been tested on its capability to act as a regenerative and antiageing product on human dermal fibroblasts (HDF), senescent fibroblast (SHDF) obtained by successive passages [20, 21] and HaCaT keratinocyte cells subjected or not to UVB light, a well-known factor implicated in premature ageing.

The results obtained show that IFC-CAF affected the morphology but not the nuclear stability of the cells used (HaCaT, HDF and SHDF). HaCaT cells treated with IFC-CAF presented a more evident polyhedral morphology typical of keratinocytes and a more basophile cytoplasm, reflecting a higher cell metabolism, compared to that of control cells. Similar effects were induced in HDF and in SHDF (Fig. 2). We have not observed evident nuclear changes after NR staining, neither in the distribution of euchromatin (by labelling acetyl-Histone H3 and trimethyl-Histone H3) nor in the nucleolar architecture (by labelling fibrillarin) even when the compound was used at a very high concentration (50 μg mL^{-1}). We have also observed that IFC-CAF did not induce an increase in the number of TUNEL-positive cells (apoptotic cells) or a significant increase in the number of positive cells for γ-H2AX (Fig. 9). Therefore, all these results indicate that IFC-CAF and the other derivative products of *C. aspersa*, SCA [8, 19], promote a better organization of the main skin cells and do not affect nuclear stability.

We have seen the distribution of HaCaT, HDF and SHDF cells in the cell cycle after treatments with IFC-CAF (Fig. 1). The treatments induced a decrease in the number of cells in G1 phase and

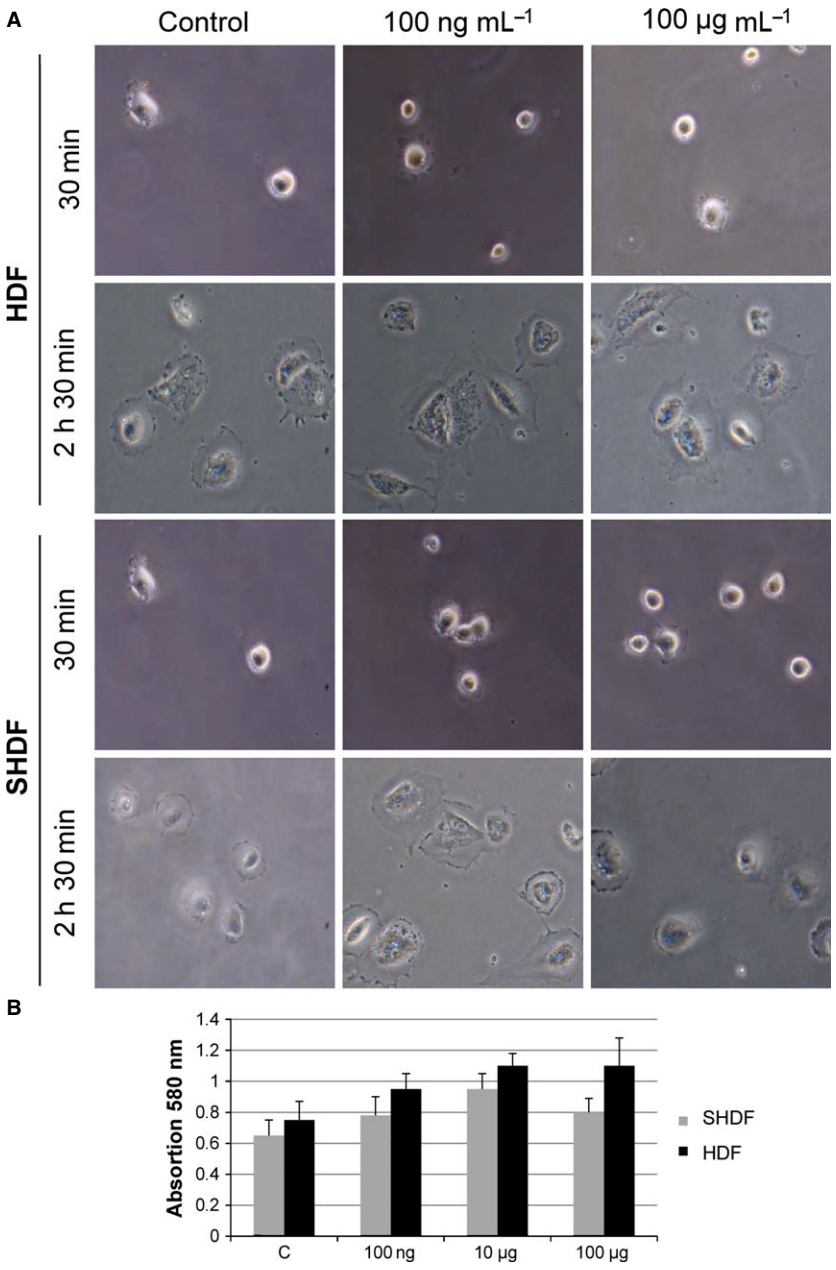

Figure 8 (A) Effect of IFC-CAF treatment (10 µg mL^{-1}) on the adhesion of HDF and SHDF. Untreated SHDF showed a delayed substrate adhesion relative to HDF. (B) IFC-CAF treatment promotes adhesion both in HDF and SHDF, both by morphology and as determined by the crystal violet assay.

an increase in S and G2-M in HaCaT cells and HDF, although these changes were not so substantial in SHDF. This increase in the number of cells in S and G2-M phases was not correlated with an increase in the total number of cells; in fact, we have seen that IFC-CAF had no proliferative effects in the tested cells at the evaluated treatment concentrations and periods. Although published results clearly demonstrated a promotion of proliferation on keratinocytes and fibroblasts when are treated with SCA [6, 8], no induction of proliferation has been described for compounds with antioxidant and regenerative properties such as resveratrol and salidroside [9, 22].

The results obtained indicated that IFC-CAF induced both fibroblast and keratinocyte migration *in vitro*. Migration of main skin cell types, keratinocytes and fibroblasts is a crucial step in the wound-healing process [23] that is impaired in ageing [24]. Cell migration involves attachment to the ECM, rearrangement of cytoskeleton and subsequent detachment of the cell from the matrix. Our results show that IFC-CAF was able to increase the

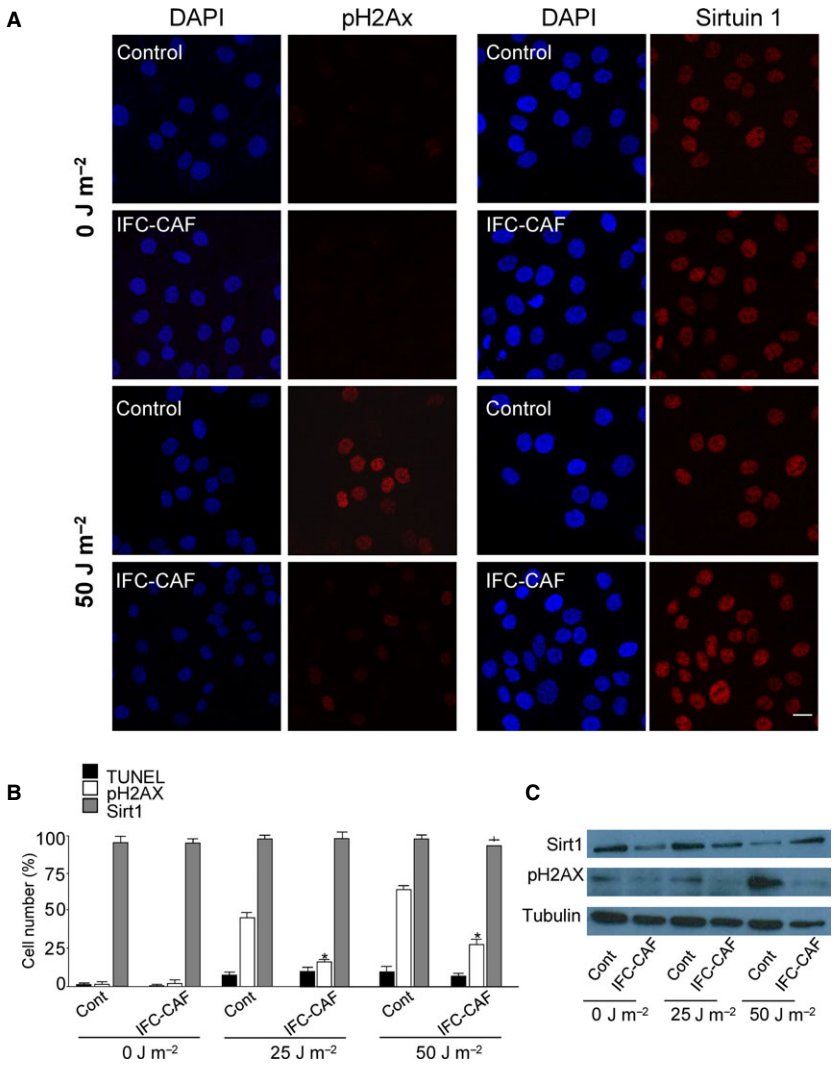

Figure 9 (A) Immunolocalization of γ-H2AX and Sirt1 in HaCaT exposed to 0 or 50 J m^{-2} of UVB under control conditions or treated with IFC-CAF. Western blot for γ-H2AX in HaCaT exposed to 0 or 50 J m^{-2} of UVB and IFC-CAF. Scale bar: 10 μm. (B) Quantification of the number of TUNEL-positive cells (apoptosis) and for the proteins: γ-H2AX and Sirt1 in HaCaT cells subjected to IFC-CAF (0) and to IFC-CAF and 25 or 50 J m^{-2} of UVB, * P<0.05 compared to Control. (C) Western blot analysis for the indicated proteins in HaCaT cells treated with IFC-CAF only or IFC-CAF and subjected to UVB radiation.

migration rate of HDF as well as keratinocytes, confirming its positive role in the wound-healing process. IFC-CAF also promotes the expression of motility-related molecules, such as vinculin, in both keratinocytes and HDF, which is a mandatory step in the process of cell migration by participating in the release and reformation of adhesive contacts with the ECM [24]. Vinculin is also implicated in survival, and an increase in the expression of this protein would be related with the activation of survival signalling pathways [24–26]. Previously, we have observed an increase in the expression of vinculin in SCA-treated HDF that would be related with the increase in the expression of molecules associated with survival such as b-catenin, FAK [8]. Therefore, from these results, we can conclude that IFC-CAF stimulated the attachment of keratinocytes and fibroblasts to the substrate favouring migration and cell survival.

In addition to the promotion of cell migration by IFC-CAF, we have also evaluated its ability as a regenerative compound in relation to the production of extracellular matrix components, particularly of FN and Col1. We have observed that the product increased the deposition of both extracellular components, which constitute a key event in tissue regeneration (Fig. 4). In keeping with those results, we have also observed that IFC-CAF significantly inhibited the production of MMP-2 and 9, enzymes that break down collagen (I, II, III and IV), the major structural component of the skin [12], suggesting that limitation of MMP expression contributes to its regenerative properties. The essential role of MMPs in skin remodelling and ageing has been demonstrated in pivotal scientific studies and products that decrease their production, including SCA, the other product isolated from *C. aspersa*, are excellent options for regenerative skin treatments [6, 8, 12].

Relating to senescent cells, it has been described that SHDF *in vitro* are much larger with round and flat appearance compared to that of HDF, produce higher amounts of vimentin and also exhibit decreased adhesion to substrate, because of a lower expression of vinculin and the presence of small focal contact sites [13, 14]. All these characteristics have been seen in our SHDF used in this work in contrast to primary HDF cells, which show higher expression of vinculin, more prominent focal adhesion contacts and high production of extracellular matrix proteins, including Col1 and fibronectin (Figs 5–7), and therefore with increased ability for movement compared with SHDF [14,27]. In addition, Schulze *et al.* [27] found an increase in stiffening with age in HDF cells obtained from human donors that could be related with changes in the organization and density of the extracellular matrix, particularly with alterations in the polymerization state and organization of actin in the cell (balance between G and F-actin). Thereby, whereas young primary cells showed relatively higher amounts of unpolymerized G-actin, the SHDF cells exhibited apparently shorter and thicker actin stress fibres because of their relatively lower levels of G-actin and higher levels of F-actin. As we have observed, the treatment with IFC-CAF had little effect on HDF, however, treated SHDF presented an expression and distribution of vimentin, F-actin and vinculin, similar to that of HDF. In addition, we have observed an increase production of fibronectin and Col1 ECM proteins that would be related with the higher migration ability of SHDF treated with IFC-CAF compared to those untreated SHDF cells. All these results are in agreement with the significantly improved adhesion and migration ability of SHDF induced by the treatment with IFC-CAF. Similar results have also been described with resveratrol in senescent fibroblasts, and the authors also related such ability with the effects induced in the reorganization of the cytoskeleton and with increased expression of protein associated with focal adhesion and spreading of fibroblasts on fibronectin [9, 12, 28]. In addition, IFC-CAF treatment decreased the number of cells that positively reacts to β-galactosidase in SHDF fibroblasts as well as decreases the expression of ageing-related proteins such as p53, p16INK4 [1, 11, 14].

Finally, we have also observed a protective effect of IFC-CAF against UVB in HaCaT cells, that could be observed as the number of positive cells for γ-H2AX decreased to reach almost control values in IFC-CAF-treated cells. The decreased expression of this protein observed by Western blot confirmed such protective effect. It should be noted that γ-H2AX constitutes an excellent biomarker of DNA damage by UV radiation [29]. The number of positive cells for sirtuin-1 (Sirt1), however, was maintained for all the evaluated conditions. Nevertheless, IFC-CAF did not increase the number of positive cells for SIRT-1. On the contrary, a decrease in the expression of this protein in all the evaluated conditions, even when the cells were only treated with IFC-CAF, was observed. Sirt1 activation has been shown to mediate protection against senescence via increased resistance to oxidative stress [30]. Several antioxidant compounds, including resveratrol, have been proved to induce its expression under different stress conditions [28]. Therefore, the results obtained in related to Sirt1 activation, should be further studied to better understand the role of IFC-CAF in the modulation of its gene expression.

From the obtained results, we can conclude that IFC-CAF ameliorated a series of functions related to cell migration, tissue repair, and attenuated age-related morphological changes of human skin cells. The use of IFC-CAF could be of particular interest as it may provide the skin benefits for improving the clinical signs of ageing.

Acknowledgements

The authors declare that the main subject of this research is subject to US patent US 5538740. The study was supported by a research grant from Industrial Farmacéutica Cantabria, S.A., Madrid, Spain. S.G. is a consultant for I.F.C.

References

1. López-Otín, C., Blasco, M.A., Partridge, L., Serrano, M. and Kroemer, G. The hallmarks of aging. *Cell* **153**, 1194–1216 (2013).
2. Watson, R.E. and Griffiths, C.E. Pathogenic aspects of cutaneous photoaging. *J. Cosmet. Dermatol.* **4**, 230–236 (2005).
3. auf dem Keller, U., Kumin, A., Braun, S. and Werner, S. Reactive oxygen species and their detoxification in healing skin wounds. *J. Investig. Dermatol. Symp. Proc.* **11**, 106–111 (2006).
4. Tsoutsos, D., Kakagia, D. and Tamparopoulos, K. The efficacy of Helix aspersa Muller extract in the healing of partial thickness burns: a novel treatment for open burn management protocols. *J. Dermatolog. Treat.* **20**, 219–222 (2008).
5. Brieva, A.G., Guerrero, A. and Pivel, J.P. Antioxidative properties of a mollusc secretion (SCA): a skin protective product. *Methods Find. Exp. Clin. Pharmacol.* **21**(Suppl A), 175 (1999).
6. Ledo, E., de las Heras, M.E. and Ledo, A. Treatment for acute radiodermatitis with Cryptomphalus aspersa secretion. *Radioprotección.* **23**, 34–38 (1999).
7. Brieva, A., Philips, N., Tejedor, R., Guerrero, A., Pivel, J.P., Alonso-Lebrero, J.L., Gonzalez, S. Molecular basis for the regenerative properties of a secretion of the mollusk Cryptomphalus aspersa. *Skin Pharmacol. Physiol.* **21**, 15–22 (2008).
8. Cruz, M.C., Sanz-Rodríguez, F., Zamarrón, A., Reyes, E., Carrasco, E., González, S. and Juarranz, A. A secretion of the mollusc Cryptomphalus aspersa promotes proliferation, migration and survival of keratinocytes and dermal fibroblasts in vitro. *Int. J. Cosmet. Sci.* **34**, 183–189 (2012).
9. Harikumar, K.B. and Aggarwal, B.B. Resveratrol: a multitargeted agent for age-associated chronic diseases. *Cell Cycle* **7**, 1020–1035 (2008).
10. Aggarwal, B.B. and Harikumar, K.B. Potential therapeutic effects of curcumin, the anti-inflammatory agent, against neurodegenerative, cardiovascular, pulmonary, metabolic, autoimmune and neoplastic diseases. *Int. J. Biochem. Cell Biol.* **41**, 40–59 (2009).
11. Merlin, J.L., Azzi, S., Lignon, D., Ramacci, C., Zeghari, N. and Guillemin, F. MTT assays allow quick and reliable measurement of the response of human tumour cells to photodynamic therapy. *Eur. J. Cancer* **28**, 1452–1458 (1992).
12. Pitozzi, V., Mocali, A., Laurenzana, A. *et al.* Chronic resveratrol treatment ameliorates cell adhesion and mitigates the inflammatory phenotype in senescent human fibroblasts. *J. Gerontol. A Biol. Sci. Med. Sci.* **68**, 371–381 (2013).
13. Fisher, G.J., Wang, Z.Q., Datta, S.C., Varani, J., Kang, S. and Voorhees, J.J. Pathophysiology of premature skin aging induced by ultraviolet light. *N. Engl. J. Med.* **337**, 1419–1428 (1997).
14. Nishio, K. and Inoue, A. Senescence-associated alterations of cytoskeleton: extraordinary production of vimentin that anchors cytoplasmic p53 in senescent human

fibroblasts. *Histochem. Cell Biol.* **123**, 263–273 (2005).
15. Dimri, G.P., Lee, X., Basile, G. *et al.* A biomarker that identifies senescent human cells in culture and in aging skin in vivo. *Proc. Natl. Acad. Sci. U. S. A.* **92**, 9363–9367 (1995).
16. Sander, C.S., Chang, H., Salzmann, S., Müller, C.S., Ekanayake-Mudiyanselage, S., Elsner, P. and Thiele, J.J. Photoaging is associated with protein oxidation in human skin in vivo. *J. Invest. Dermatol.* **118**, 618–625 (2002).
17. Capote, R., Alonso-Lebrero, J.L., Garcia, F., Brieva, A., Pivel, J.P. and Gonzalez, S. Polypodium leucotomos extract inhibits trans-urocanic acid photoisomerization and photodecomposition. *J. Photochem. Photobiol., B* **82**, 173–179 (2006).
18. Farris, P. Idebenone, green tea, and Coffeeberry extract: new and innovative antioxidants. *Dermatol. Ther.* **20**, 322–329 (2007).
19. Mukherjee, S., Date, A., Patravale, V., Korting, H.C., Roeder, A. and Weindl, G. Retinoids in the treatment of skin aging: an overview of clinical efficacy and safety. *Clin. Interv. Aging* **1**, 327–348 (2006).
20. Hayflick, L. and Moorhead, P.S. The serial cultivation of human diploid cell strains. *Exp. Cell Res.* **25**, 585–621 (1961).
21. Kuilman, T., Michaloglou, C., Mooi, W.J. and Peeper, D.S. The essence of senescence. *Genes Dev.* **24**, 2463–2479 (2010).
22. Mao, G.X., Wang, Y., Qiu, Q. *et al.* Salidroside protects human fibroblast cells from premature senescence induced by H2O2 partly through modulating oxidative status. *Mech. Ageing Dev.* **131**, 723–731 (2010).
23. Clark, R.A.F. Cutaneous tissue repair: basic biologic considerations. I. *J. Amer. Acad. Dermatol.* **13**, 701–725 (1985).
24. Reed, M.J., Ferara, N.S. and Vernon, R.B. Impaired migration, integrin function, and actin cytoskeletal organization in dermal fibroblasts from a subset of aged human donors. *Mech. Ageing Dev.* **122**, 1203–1220 (2001).
25. Webb, D.J., Donais, K., Whitmore, L.A., Thomas, S.M., Turner, C.E., Parsons, J.T. *et al.* FAK-Src signalling through paxillin, ERK and MLCK regulate adhesion disassembly. *Nat. Cell Biol.* **6**, 154–161 (2004).
26. Giancotti, F.G. and Ruoslahti, E. Integrin signaling. *Science* **285**, 1028–1033 (1999).
27. Schulze, C., Wetzel, F., Kueper, T. *et al.* Stiffening of human skin fibroblasts with age. *Clin. Plast. Surg.* **2012**, 9–20 (2012).
28. Farris, P., Krutmann, J., Li, Y.H., McDaniel, D. and Krol, Y. Resveratrol: a unique antioxidant offering a multi-mechanistic approach for treating aging. *J. Drugs Dermatol.* **12**, 1389–1394 (2013).
29. Cleaver, J.E. γH2Ax: biomarker of damage or functional participant in DNA repair "all that glitters is not gold!". *Photochem. Photobiol.* **87**, 1230–1239 (2011).
30. Guarente, L. Sirtuins, aging, and metabolism. *Cold Spring Harb. Symp. Quant. Biol.* **76**, 81–90 (2011).