

Extract From the Leaves of *Polypodium Leucotomos* Stimulates DNA Repair Signals γ H2AX and P53 to Prolong the Keratinocyte DNA Repair Phase of the Cell Cycle

ALBERTO GANDARILLAS, ANTONIO GUERRERO, ARANCHA DELGADO-RUBIN, SALVADOR GONZÁLEZ, AZAHARA RODRÍGUEZ-LUNA AND LUCÍA SAN JUAN
Cell Cycle, Stem Cell Fate and Cancer Laboratory, Institute for Research Marqués de Valdecilla (IDIVAL), Santander, Spain (A.Ga., L.S.J.)

INSERM, Occitanie Méditerranée, Montpellier, France (A.Ga.)

Innovation and Development, Cantabria Labs, Madrid, Spain (A.Gu., A.D.R., A.R.L.)

Department of Medicine and Medical Specialties, Alcalá de Henares University, Madrid, Spain (S.G.)

Faculty of Health Sciences, Universidad Loyola Andalucía, Seville, Spain (A.R.L.)

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Synopsis

Polypodium leucotomos extract (PLE) has been extensively utilized by both topical and oral administration due to its beneficial protective or repair effects on various cell types of the skin. Now, we have investigated the direct function of PLE on the proliferative capacity and the cell cycle of freshly isolated keratinocytes from human skin. To note, PLE significantly induced the phosphorylation of histone H2AX, an early signal that triggers the DNA repair machinery upon genetic damage, both in exponentially proliferating cells and upon ultraviolet light irradiation. Interestingly, the induction of the signal was independent of actual DNA breaks, as monitored by comet assays. PLE also induced p53, the “guardian of the genome” that halts cells in the G2 phase of the cell cycle to allow DNA repair. As a result, epidermal multipotent stem cells grew more slowly but were more protected as they displayed a more powerful amplification after the PLE treatment. We discuss a model of action of PLE on the keratinocyte cycle that might be common to other natural substances used, or with a potential to be used, in skin cosmetics.

INTRODUCTION

The skin is the first barrier in the defense against ultraviolet (UV) radiation from sunlight. Among other cellular damages, including the formation of oxygen and nitrogen reactive species, inflammation or immunosuppression UV light causes genetic damage.^{1–3} These alterations eventually contribute to aging and cancer. DNA damage and immunosuppression are crucial factors in photocarcinogenesis, as they tilt the scales in favor of two central hallmarks of cancer, DNA mutation and immune evasion.⁴ Not only is the epidermis continuously exposed to UV radiation, but it undergoes continuous cell renewal, thus

*Address all correspondence to Alberto Gandarillas, agandarillas@idival.org

increasing the risk of genetic alterations. Hence, skin protection against UV light can be considered preventive medicine, as there becomes a need toward preventing short-term (erythema, inflammation) and long-term (cancer, aging) effects of exposure to this harmful radiation. Epidermal keratinocytes are a major target of radiation-induced damage as they make up most epidermal cells.

In the last decades, the public has become widely aware of the risks of sustained exposure to sunlight, and the use of sun-protection products has significantly increased. Diverse natural products have been used to counteract UV radiation-induced skin damage through different approaches. One is the use of sunscreens, which prevent high-energy photons from reaching sensitive biological targets, e.g., nuclear DNA; these substances possess the ability to absorb photonic energy, thereby preventing biological substrates from doing it. Another possibility is to use substances that do not act as a sunscreen but minimize injury through alternative mechanisms, e.g., attenuating oxidative or DNA damage. These substances do not necessarily need to be applied topically, but they can also be administered orally.⁵

Our group has focused on the beneficial effects of a hydrophilic extract from the leaves of the fern *Polypodium leucotomos* (PLE, Fernblock®). PLE has a remarkable safety profile (Nestor et al., 2015), has a function as a barrier (it absorbs UV photons), and acts as a biological modifier.^{6,7} However, its main beneficial effect is due to its extraordinary antioxidant ability and protective cellular effects. These include improved myeloid (Langerhans) cell survival upon UV irradiation, decreased trans-urocanic acid isomerization and increased DNA repair enzyme activity.^{8–11} PLE is currently used in topical and oral sunscreen formulations.¹² It also counteracts other deleterious effects of UV irradiation, e.g., photoaging and photoimmunosuppression.^{13,14} Its mechanism of action seems multipronged, but its antioxidant capability plays a fundamental role in its beneficial effects.⁹

Although the above beneficial effects are well documented, the genetic protective mechanisms of PLE in the keratinocyte biology have not been addressed yet. Unrepaired DNA damage blocks proliferation and induces differentiation of keratinocytes of the diverse stratified epithelia of the skin or head and neck.^{15–17} This response is controlled by mitotic checkpoints.^{16,18–20} The differentiation response protects keratinocytes from apoptosis even upon moderate UV irradiation.²¹ The G2 phase of the cell cycle, prior to mitosis, is a major phase for DNA repair. Upon DNA damage, G2 checkpoints halt the cycle to ensure that DNA is repaired before cell division.²² Multipotent stem cells within the basal layer of the epidermis maintain the tissue and are targets of genetic insult and aging.^{23,24} These cells are not totipotent but are capable of replenishing the tissue and giving rise to different progenitors.

We have investigated the effects of treating primary human keratinocytes with PLE on DNA damage and the cell cycle before or after UV irradiation. Interestingly, epidermal keratinocytes treated with PLE were retarded in growth but conserved a greater capacity to amplify after treatment. We have investigated the molecular mechanisms underlying this protective effect. A “soft arrest” in G2/M upon PLE treatment might allow cells to better repair their DNA before undergoing mitosis. PLE appears to achieve this by enhancing cellular DNA repair signals. This mechanism might contribute to preserve genome integrity and the proprieties of epidermal stem cells face to DNA replication stress or UV irradiation. We discuss the implications for skin cancer and aging.

RESULTS

PLE SLOWS DOWN GROWTH AND PROTECTS THE AMPLIFYING CAPACITY OF HUMAN EPIDERMAL KERATINOCYTES

We cultured freshly isolated keratinocytes from human epidermis in Rheinwald conditions.²⁵ In these conditions, cells proliferate, stratify, and differentiate. To investigate the effect of PLE on the clonal growth capacity of epidermal stem cells, we performed clonogenicity assays with human isolated primary keratinocytes of the skin. These assays at low density allow monitoring the growth of epidermal stem cells since they are the only cells capable to give rise to large proliferative colonies.²⁶ Proliferative progenitors are committed to differentiate after a small number of divisions and give rise to small differentiated colonies. Primary keratinocytes cultured in the presence of PLE for 8 days (0.8 or 1.6 mg/ml) displayed a significantly greater reduction in the size of the colonies than controls (Figure 1A). When we examined the cultures after incubation with PLE for 8 days, we observed that cells were slightly larger and less densely packed than controls (Figure 1B). The smaller size of the colonies upon PLE might be caused by a toxic effect or by retarded growth. To answer this question, we plated cells for clonogenicity assays after treatment, in the absence of PLE, to test for the later effects on the capacity of stem cells. Interestingly, PLE-treated keratinocytes displayed a significant increase in the number of colonies (Figure 1C).

PLE DELAYS PROGRESSION OF KERATINOCYTES THROUGH THE G2 PHASE OF THE CELL CYCLE AND INDUCES DNA REPAIR SIGNALS γ H2AX AND P53 INDEPENDENTLY OF DNA DAMAGE

The growth inhibitory effect of PLE suggested that it was acting on the keratinocyte cycle. Therefore, we examined the effect on the cell cycle. As before, we treated human keratinocytes with PLE (0.8 mg/ml) for 8 days and determined their DNA content by flow cytometry. We observed a striking change in the cell cycle upon PLE. G1 was significantly diminished and G2/M was augmented (Figure 2A). We found a similar accumulation of cells in G2/M after various periods of treatment with PLE, from 5 to 9 days (not shown). Considering that cells proliferated more slowly, this would not indicate a cell cycle activation but rather, a delay of progression through the G2/M transition. We observed no signs of apoptotic sub-G1 cells in the DNA content analyses, further indicating that the growth inhibitory effect of PLE was due to a slower cell cycle. An increase in forward scatter and side scatter was also observed within the proliferative region, consistent with the morphology displayed in Figure 1 and with the typical cell size increase in G2/M due to the cellular growth that takes place prior to cell division (Figure 2B).^{20,26}

Since keratinocytes were more clonogenic after the PLE treatment, we investigated the expression of γ H2AX by flow cytometry, a central signal in the induction of the DNA repair cascade.²⁷ Interestingly, upon a similar PLE treatment as before, we observed a significant increase in the phosphorylation of H2AX (γ H2AX; Figure 3A and B). Since γ H2AX induces the G2/M checkpoint to allow DNA repair, in part via the p53, we analyzed the expression of this transcription factor by Western blotting and immunofluorescence (Figure 3C and D). PLE treatment significantly induced the expression of p53 to levels comparable to those induced by treatment with the genotoxic drug Doxorubicin (Figure 3C).

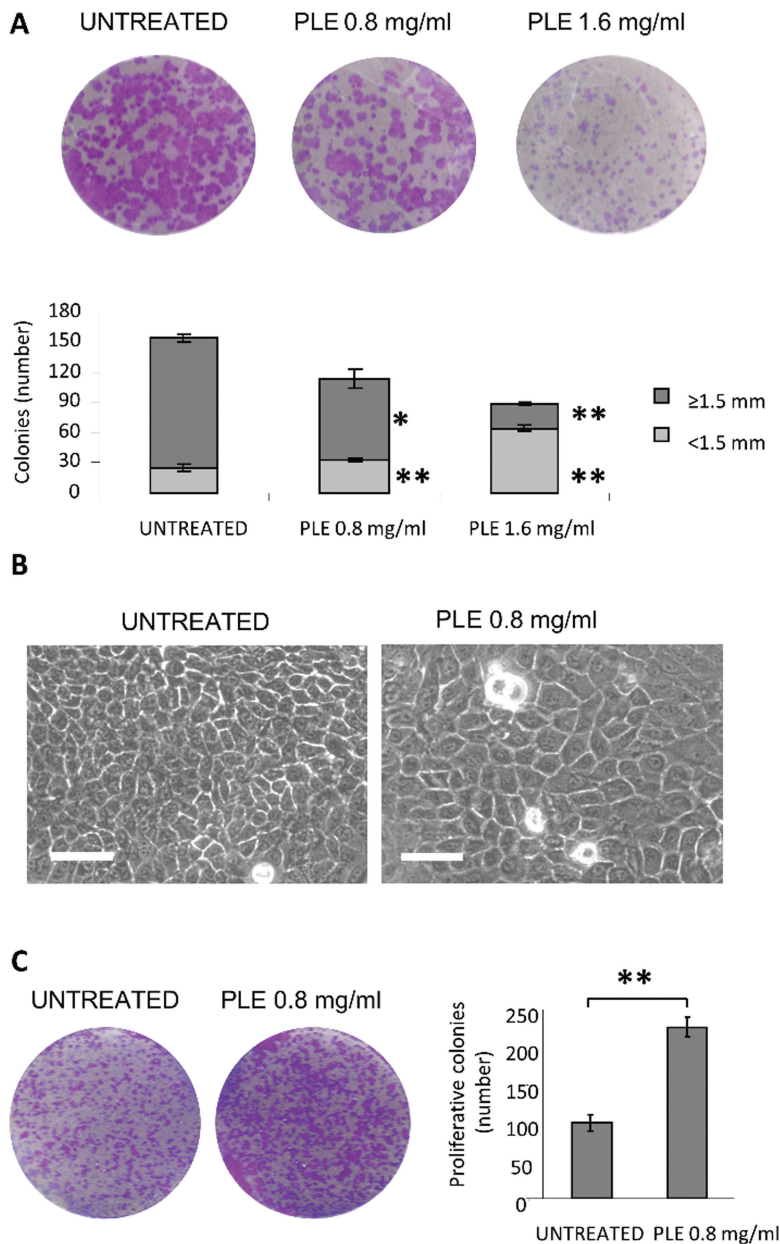


Figure 1. PLE slows down growth and protects the amplifying capacity of human epidermal keratinocytes. (A) Representative images of clonogenicity assays of keratinocytes cultured at low density for 10 days in the presence or absence of PLE as indicated. Histogram: quantification of the number of colonies in A, grouped by size; (B) phase contrast images showing the representative morphology of keratinocytes cultured in the presence of PLE (0.8mg/ml). Scale bar: 100 μ m; and (C) representative clonogenicity assays of keratinocytes cultured or not in the presence or absence of PLE (0.8 mg/ml) for 8 days as indicated, then seeded for clonogenicity assays as in A, in the absence of PLE. Data are mean $\pm$ SD of duplicate or triplicate samples, representative of two to three independent experiments.

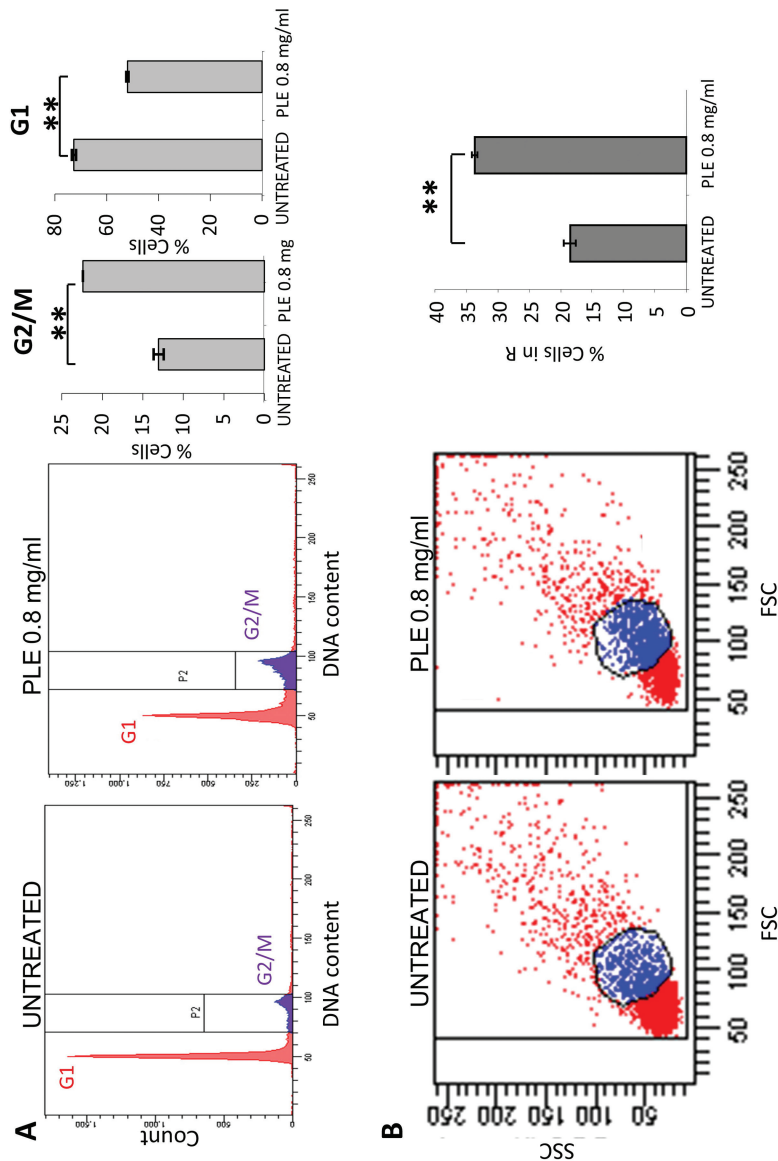


Figure 2. PLE delays progression of epidermal keratinocytes through the G2/M phase of the cell cycle. (A) Representative flow cytometry analyses of DNA content determined by propidium iodide (PI) staining of cultured primary keratinocytes, treated or not with PLE for 8 days as indicated. Quantitation of the percent of cells in the G1 or the G2/M phase of the cell cycle is shown on the right histograms; (B) representative flow cytometry analyses of light scatter parameters of cultured primary keratinocytes, treated or not with PLE for 8 days, as indicated. The black circle corresponds to proliferative cells displaying a larger size typical of cells in the DNA repair (R) G2/M phase of the cell cycle (cells are twice as big). Quantitation of cells in this region is shown on the right. Data represent mean $\pm$ SD of duplicate samples, representative of three independent experiments. $**p < 0.01$. SSC: Side Scatter; FSC: Forward Scatter.

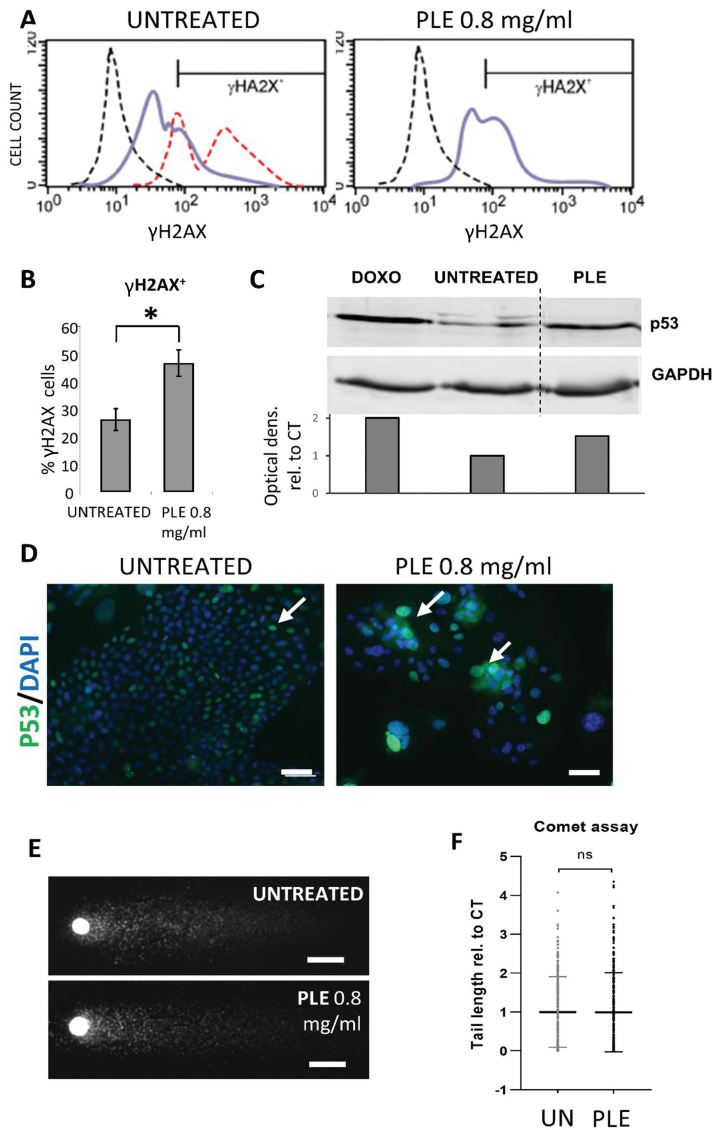


Figure 3. PLE induces DNA repair signals γ H2AX and p53 in epidermal keratinocytes independently of DNA damage. (A) Representative flow cytometry profiles showing levels of γ H2AX (solid magenta line) in primary human keratinocytes, untreated or treated with PLE for 8 days. As a positive control of γ H2AX induction, a separate sample of cells was treated for 24 hours with 1 μ M doxorubicin (dotted red line). Dotted black line is negative isotype antibody control; (B) quantitation of the percent of γ H2AX-positive cells according to the gate shown in A; (C) Western blot for p53 expression in cells untreated or treated with PLE for 8 days. Cells treated with Doxorubicin as in A were included as a control for the induction of p53. GAPDH is used as protein-loading control. Bottom histogram represents the optical density normalized to GAPDH and relative to control (CT); (D) immunofluorescence for expression of p53 (arrows) of cells treated for 5 days as indicated. Scale bar: 50 μ m. (E) representative nuclear image of comet assay analyses of human epidermal keratinocytes untreated or treated with PLE for 6 days, as in A and B, to test for chromosomal fragmentation; and (F) quantitation of DNA fragmentation measured by tail length, relative to untreated (UN) control cells ($n = 299/302$). Scale bar: 10 μ m. Data are mean $\pm$ SD of duplicate samples representative of two independent experiments. * $p < 0.05$; ns: not significant ($p > 0.05$).

Note that γ H2AX and p53 are central cellular signals responding to DNA damage to induce DNA repair. Treatment of PLE was not expected to induce DNA damage given its cellular protective effects shown above and elsewhere. An interesting alternative was that PLE induces the signals independently of the damage, therefore boosting DNA repair. To test this possibility, we performed comet assays to analyze actual nuclear chromosomal fragmentation. As shown in Figure 3E and F, the same doses of PLE that induced γ H2AX (0.8 mg/ml) did not cause an increase in DNA fragmentation. This indicates that the significant boost of the DNA repair signals was independent of DNA damage.

PLE ATTENUATES THE DIFFERENTIATION RESPONSE INDUCED BY REPLICATION STRESS OR SUBLETHAL UV IRRADIATION

Since in keratinocytes replication stress via DNA damage triggers terminal differentiation during proliferation, we analyzed the expression of epidermoid differentiation markers involucrin and keratin K16.^{28–30} Involucrin is a precursor of the cornified envelope, and K16 is expressed in the skin during hyperplasia or other stress conditions.³¹ PLE reduced the proportion of cells expressing involucrin and keratin K16 as analyzed by flow cytometry and immunofluorescence (Figure 4A–C).

To evaluate the protective effect of PLE on skin keratinocytes on the face to UV light, we pretreated primary keratinocytes with PLE for 6 days and then irradiated them. Pretreatment with PLE had only a small effect on the cell cycle profile of irradiated cells (Figure 5A). Interestingly, the cell cycle profile of irradiated cells was very similar to the profile induced by PLE in nonirradiated cells (compare with Figure 2A). PLE however, induced moderately further the levels of γ H2AX in irradiated cells (Figure 5B). As mentioned above, keratinocytes respond to unrepaired DNA damage by initiating terminal differentiation (unless exposed to acute doses of UV irradiation, which induces apoptosis). Consistently, sublethal UV irradiation induces keratinocyte differentiation.²¹ To note, treatment with PLE diminished the proportion of UV-induced terminally differentiating cells, according to involucrin expression and cellular morphology (Figure 5C and D).

DISCUSSION

PLE REINFORCES CELL CYCLE CONTROLS

We have shown cumulative evidence indicating that PLE protects epidermal cells from proliferative or UV-induced DNA damage by slowing down the keratinocyte cell cycle at the G2/M transition. G1 and G2 are the major points of the cell cycle where DNA repair is achieved.^{22,32} Moreover, in keratinocytes, the G2/M checkpoints control cell fate whether to divide, to differentiate, or to undergo apoptosis.^{20,28} Unrepaired cells that enter mitosis prematurely will differentiate. The cell cycle speeds up due to proliferative stimuli such as the *in vitro* conditions that we have implemented in this study or upon stimuli such as UV light.^{21,28} Stem cells, when activated, give rise to rapidly proliferative progeny. These cells are already committed to differentiate terminally due to the loss of cell cycle control, replication stress, and DNA damage.

The γ H2AX signal and the transcription factor p53 both trigger the G2 checkpoints and halt cells in G2 to promote DNA repair.^{22,27,33} These signals respond to DNA damage.

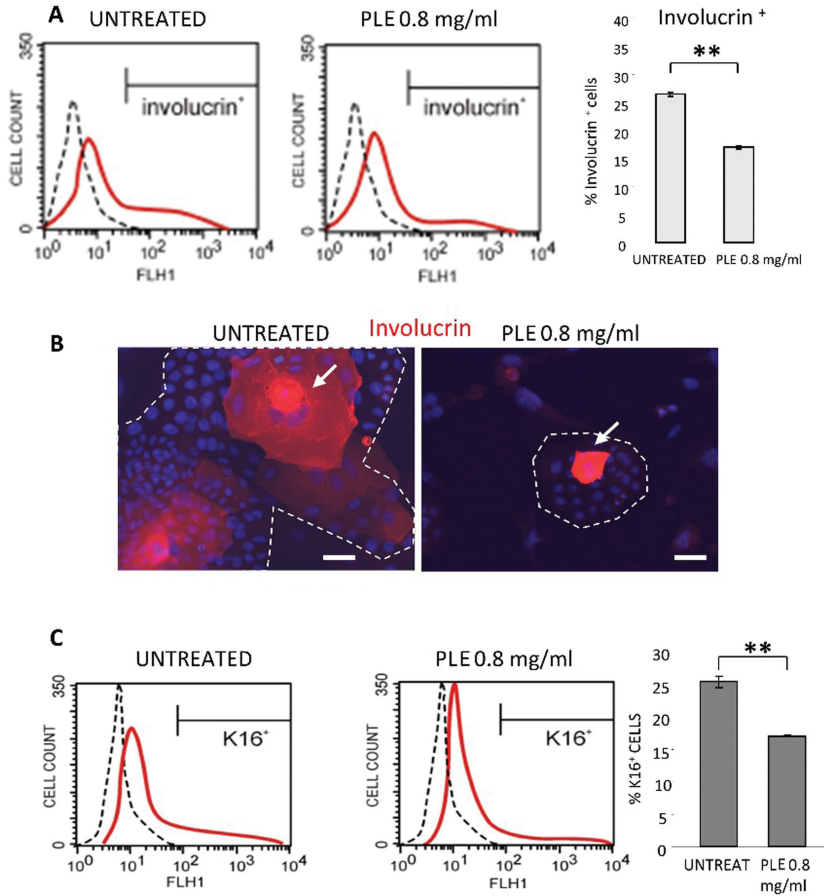


Figure 4. PLE reduces the proportion of keratinocytes undergoing stress-induced terminal differentiation. (A) Left, representative flow cytometry profiles of the levels of involucrin (solid red line) in primary human keratinocytes treated or not with PLE, as indicated, for 8 days. Dotted black line is negative antibody control. Right, quantification of the percent of involucrin-positive cells according to the gate shown on the left. Data are mean $\pm$ SD of duplicate samples representative of two independent experiments. $**p < 0.01$; (B) representative immunofluorescence images of untreated or PLE-treated keratinocytes, as indicated, for 5 days. Red is involucrin (arrows) and blue is nuclear DNA stained with 4',6-diamidino-2-phenylindole. Broken line outlines the border of the colonies. Scale bar: 50 μ m; and (C) representative flow cytometry profiles of keratin K16 expression (red line) in human keratinocytes treated or not with PLE for 8 days. Dotted line as in A. Bar histogram shows the percent of K16-positive cells according to the gate in the left plots. Data are mean $\pm$ SD of duplicate samples. $**p < 0.01$. FLH1: Fluorescence channel 1 Height.

However, in our hands the treatment with PLE induced these signals without a detectable increase of actual DNA damage. This indicates that PLE is capable of boosting the G2 DNA repair machinery in a direct manner by mechanisms still to be elucidated. The p53 is referred to as the guardian of the genome due to its capacity to correctly coordinate the cell cycle phases. Altogether, our results indicate that PLE reinforces the orchestral execution of the cell cycle. This is consistent with the lower index of expression of keratin K16, a marker of epidermal hyperproliferation and stress.³⁴

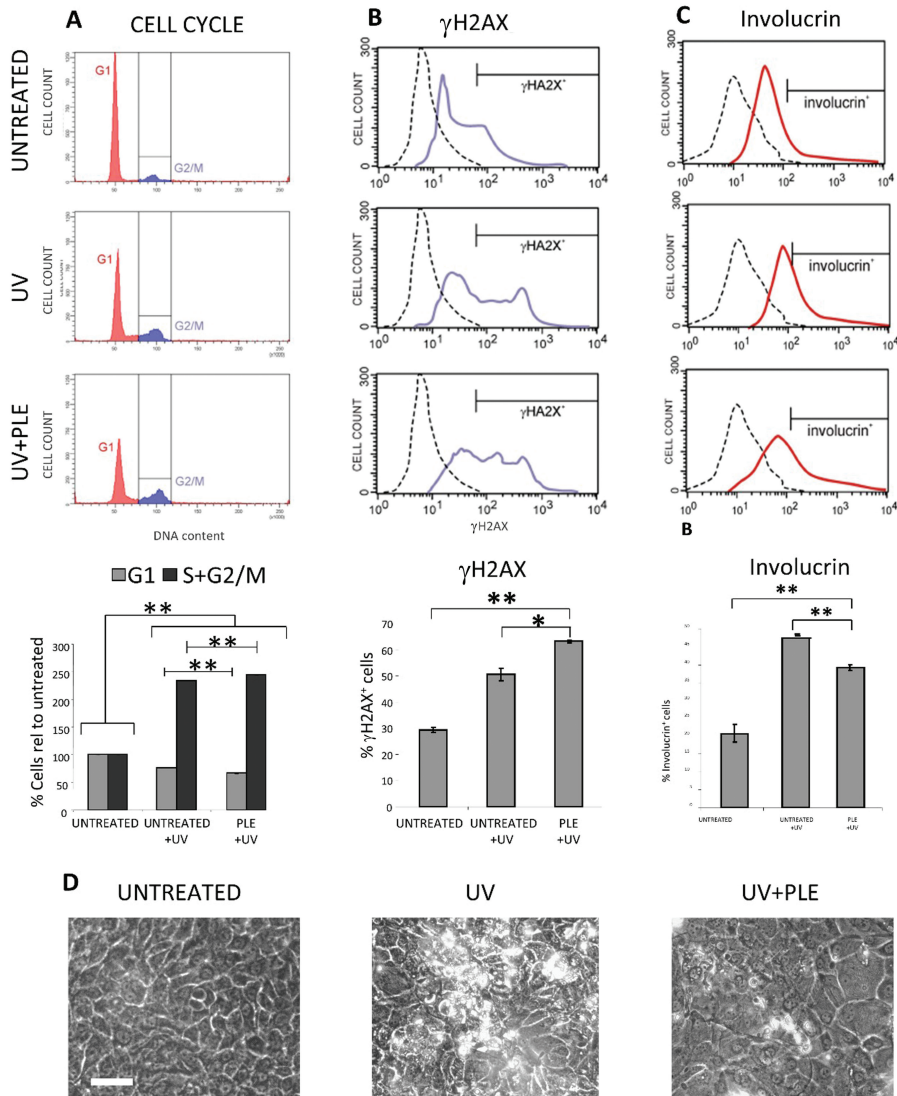


Figure 5. Pretreatment with PLE boosts the γ H2AX signal and attenuates the differentiation response induced by sublethal UV irradiation. (A) Top, flow cytometric analyses of DNA content determined by propidium iodide (PI) of epidermal keratinocytes, treated or not, as indicated, with PLE for 6 days and irradiated with sublethal doses (25 mJ/cm²) of UVB light. Cells were analyzed 48 hours postirradiation. Bottom, bar histogram shows the quantification of the percentage of cells in G1 and S + G2/M normalized to untreated, nonirradiated cells; (B) top, flow cytometry profiles of the levels of γ H2AX (solid magenta line) in epidermal keratinocytes treated as in A. Secondary antibody staining control is shown as a dotted black line. Bottom, quantification of percent of γ H2AX-positive cells according to the gate shown on the plots above. Data are mean $\pm$ SD of duplicate samples representative of two to three independent experiments. * p < 0.05, ** p < 0.01; (C) flow cytometry profiles of the levels of involucrin (solid red line) in epidermal keratinocytes as in A. Secondary antibody staining control is shown as a dotted black line. Bottom: bar histogram: quantification of the percent of involucrin-positive cells according to the gate on the plots above. Data are mean $\pm$ SD of duplicate samples representative of two independent experiments. ** p < 0.01; and (D) photographs of phase contrast microscopy of cells in A–C, as indicated. Note the differentiated phenotype in the UV-treated culture, with large cells and shedding, compared with the less differentiated culture when pretreated with PLE. Scale bar: 50 μ m.

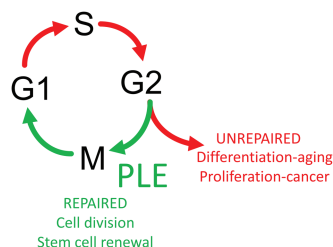


Figure 6. Proposed model for the protective effect of PLE on the epidermal cell cycle and renewal. By slowing down the cell cycle at the G2 phase, PLE provides keratinocytes with more time to achieve DNA repair, therefore protecting the integrity and renewal capacity of stem cells.

PLE ATTENUATES THE DIFFERENTIATION RESPONSE TO DNA DAMAGE

Epidermal stem cells are able to give rise to various lineages and have a great capacity of amplification *in vitro*.^{1,23,25,35} Epidermal stem cells are targets of genetic damage and aging. Allowing more time in the DNA repair G2 phase should prolong the renewal capacity of stem cells, and this is what we observed when cells were released after PLE treatment. The boost of the DNA repair signal also should diminish the proportion of cells undergoing differentiation postmitotically. Premature keratinocyte differentiation eventually contributes to skin aging. Cells treated with PLE were more protected against aging, as they retained a greater capacity for self-renewal and amplification after treatment. Consistently, we observed an attenuated rate of terminal differentiation in both exponentially growing cells and UV-irradiated cells that were treated with PLE. UV light and aging are the main causes of skin epithelial cancer. The mechanism we describe might support the beneficial anticancer activity of the PLE in response to UV radiation.⁵

A MECHANISM POTENTIALLY COMMON TO OTHER SOLAR PROTECTIVE COMPOUNDS

The model we draw might be common to other natural substances protecting cells from UV light (Figure 6). This is in line with observations that many antioxidant agents inhibit cell proliferation in a concentration-dependent manner, e.g., N-acetyl cysteine or green tea polyphenols.^{36,37} The effects on the cell cycle described here show that PLE has a multipronged function in protecting cellular DNA by controlling the cell cycle and favoring repair and checkpoint assessment. Future studies will elucidate the specific mechanisms and signaling cascades by which PLE and possibly other natural substances protect the epidermal cell cycle.

MATERIALS AND METHODS

KERATINOCYTE CULTURE

Primary human keratinocytes were isolated from neonatal foreskins of two different individuals and cultured Rheinwald conditions in the presence of a fibroblastic feeder layer of J2-3T3 cells (inactivated by 6 µg/ml mitomycin C) in 5% fetal calf serum–FAD medium (1,6 mM — “high” — calcium), as described previously; 4×10^5 cells were plated per 10-cm dish. J2-3T3 cells were cultured in DMEM containing 10% calf serum, 2 mM L-Glutamine, and 100 U/mL penicillin/streptomycin.^{25,35}

Fernblock® is a standardized hydrophilic extract from the leaves of *P. leucotomos* that has been developed to take advantage of the photoprotective properties of ferns by providing a consistent phenolic content.³⁸ It was obtained as lyophilized powder from Cantabria Labs (Madrid, Spain). The extract was stored at room temperature (RT) and shielded from light, following the provider's instructions. Stock solutions were prepared in distilled water under agitation at 25°–30°C. Defrosted for every assay, PLE was diluted into the culture medium to the final desired concentrations. We first tried various concentrations from 0,8 to 2,4 mg/ml and chose the minimal dose with a similar effect (0,8 mg/ml). In addition, we treated cells for various lengths of time, from 5 to 10 days, with consistent results. Treatments were extended up to 10 days for assays at low cell density (clonogenicity). Chosen time of treatment was shorter for rapid responses or immunofluorescence on individual colonies, 5 to 8 days, depending on the growth pace of control cells.

UV IRRADIATION

UV irradiation was carried out with a UPV CL-1000 Series UV crosslinker (Krackeler Scientific, Albany, NY) with fluorescent tubes of 312 nm shortwave ultraviolet B (UVB). Keratinocytes plated in 60-mm dishes were irradiated in subconfluence conditions (70% of the plate) at a (sublethal) dose of 25 mJ/cm². At 14 hours before irradiation, culture medium was replaced to achieve cell cycle consistency. To irradiate the plates, medium was removed and replaced with tempered phosphate-buffered saline (PBS). Immediately after irradiation, PBS was removed, and fresh culture medium was added to the plates. Nonirradiated control cultures were subjected to the same procedure except for the UV irradiation.

CLONOGENICITY ASSAY

For clonogenicity assays, primary keratinocytes were cultured in FAD medium at very low density (2,500 cells per T6 well) in duplicate or triplicate samples. Mitomycin-inactivated 3T3-J2 fibroblasts were always plated as feeder layer. About 14 days later, cultures were washed twice with PBS and fixed in 4% formaldehyde for 5 minutes at RT. After additional rinses with PBS, the cultures were stained for 10–15 minutes at RT with Rhodanile blue, as described before.³⁹ With this mixture, keratinocyte colonies are dyed in pink, while background feeders are stained in blue. Visible colonies (i.e., >0.4 mm in diameter, approximately 25–30 cells) were scored on every dish.

FLOW CYTOMETRY ANALYSIS

Primary human keratinocytes were analyzed for the cell cycle and differentiation by flow cytometry as described.⁴⁰ Briefly, cells were harvested and fixed in cold 70% ethanol for 30 minutes at 4°C (for cell cycle, γH2AX, and keratin-16 analysis) or with 3,7% paraformaldehyde in PBS for 15 minutes at RT (for involucrin analysis). For cell cycle analyses, cells were incubated with 1 mg/ml propidium iodide (PI) (P3566, ThermoFisher Scientific, Waltham, MA), 20 mg/ml RNase (12091-021, ThermoFisher Scientific) for 16 hours at 4°C.

For protein analysis, the following primary antibodies were used: mouse antihistone H2AX-ser 139 (JBW301, 05636i, MilliporeSigma, Burlington, MA); mouse antikeratin 16 (LL025, sc-53255, Santa Cruz Biotechnology, Dallas, TX); mouse anti-involucrin (sy3, lab-made, Hudson, 1992). All antibody stainings were controlled using similar concentration of isotype-negative antibody (mouse IgG, Sigma-Aldrich). Alexa Fluor® 488-conjugated goat anti-mouse IgG antibody was used as secondary antibody (115-547-003, Jackson ImmunoResearch, West Grove, PA). After staining, samples were resuspended and filtered through a 70- μ m pore nylon mesh (NY-0130/NYLON, Labopolis, Madrid, Spain) to prevent the presence of aggregates. Analyses were carried out on a CytoFLEX cytometer (Beckman Coulter, Brea, CA). Ten thousand cells were gated and acquired.

IMMUNOFLUORESCENCE AND WESTERN BLOTTING

For immunofluorescence, primary human keratinocytes were cultured in 10-mm round coverslips. Cells were fixed at RT with cold MeOH for 10 minutes or fixed with 3.7% paraformaldehyde in PBS for 10 minutes at RT and then permeabilized with cold MeOH. Staining was performed as previously described.¹⁶ The following primary antibodies were used: mouse anti-involucrin (sy3, lab-made, Hudson, 1992) and rabbit anti-p53 (FL393, SC-6243, Santa Cruz Biotechnology). Alexa Fluor® 488-conjugated goat anti-mouse or anti-rabbit IgG antibody were used as secondary antibodies (115-547-003, Jackson ImmunoResearch). DNA was stained with 0.2 μ g/ml 4',6-diamidino-2-phenylindole. Coverslips were mounted with ProLong Gold Antifade Reagent (P10144, ThermoFisher Scientific). Images were acquired with a fluorescent microscope (Zeiss, Oberkochen, Germany) and processed using Axiovision or Zen Blue software.

For Western blotting, cellular pellets were lysed and subjected to SDS-PAGE electrophoresis and blotting as previously described (soluble protein fraction).¹⁶ Rabbit anti-p53 (FL393, SC-6243, Santa Cruz Biotechnology) was used for p53 detection. An equal amount of protein was loaded onto the gel, and glyceraldehyde-3-phosphate dehydrogenase (GAPDH) was used as loading control. Secondary HRP-conjugated goat anti-rabbit or anti-mouse IgG antibodies (170-6516, 170-6515, Bio-Rad, Hercules, CA) were used. Membranes were incubated with enhanced chemiluminescence substrate (Amersham ECL Prime Western Blotting Detection Reagent, RPN2232, Cytiva, Marlborough, MA) following supplier's instructions and imaged with FUSION Solo S-Western Blot & Chemi Imaging system (Vilber Lourmat, Eberhardzell, Germany).

COMET ASSAYS

Single-cell electrophoresis, or comet assays, were performed as previously described.²⁸ Briefly, cells were gently harvested in the dark and embedded in low-melting-point agarose. Then a thin layer of the cell suspension was added to slides previously coated with normal-melting-point agarose. After solidification, slides were incubated for 1 hour in cold lysis solution (10% DMSO, 1% Triton X-100) and 89% lysis buffer (NaCl 2.5 M, EDTA 100 mM, Tris 10 mM, NaOH 200 mM, pH 10). Then electrophoresis was carried out and the samples were fixed with absolute ethanol and stained with RedSafe 1:500 (21141, Intron Biotechnology, Seongnam, South Korea). Images were acquired with a Zeiss

fluorescent microscope and analyzed with ZEN 2012 program (Zeiss). DNA fragmentation was quantitated according to comet tail length.

STATISTICS

Data are mean $\pm$ SD from at least two culture independent dishes conditions representative of two or three independent experiments, unless stated otherwise. *P* values were calculated with two-tailed Student's *t*-test (Excel). *P* values of <0.05 were considered significant. Most analyses were repeated in two different epidermal strains from two different individuals with similar results.

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CONFLICT OF INTEREST

S.G. is a consultant for Cantabria Labs, and A.Gu., A.D.R., and A.R.L. belonged to the Innovation and Development Department at Cantabria Labs (at the time of the work), which produced Fernblock® technology. The remaining authors declare no conflict of interest.

CONTRIBUTIONS

A.G.: data curation, experimental design, analyses, funding, figure making, manuscript writing, and validation; A.Gu. and A.D.R.: experimental design, analyses, funding, manuscript validation; S.G.: analyses, manuscript validation; A.R.L.: analyses, manuscript writing, and validation; L.S.J.: data curation, figure making, manuscript writing, and validation.

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