N. Belot*, C. Bastin, M. Leroy, R. Fandango, L. Naa, A . Chretien, O. Toussaint, A. Meybeck, M. Salmon*

UV-Induced Premature Senescence as a Model to Study Photo-Aging: A Case Study with 1,25 Dihydroxyvitamin D3

Introduction

It is known that many proliferative cell types like lung or skin human diploid fibroblasts (HDFs), melanocytes, endothelial cells, retinal pigment epithelial cells, erythroleukemia cells, exposed to subcytotoxic stress (UV, organic peroxides, H_2O_2, ethanol, mitomycin C, hyperoxia, etc.), undergo stress-induced premature senescence or SIPS which is closely related to replicative senescence (1, 2). SIPS can be defined as the long term effects of subcytotoxic stress on proliferative cell types, including irreversible growth arrest of a majority of the cell population. SIPS is characterized by several biomarkers that are common to cells having reached the limit of their proliferative life span. In particular, the proportion of HDFs positive for senescence-associated β-galactosidase (SA β-gal) activity increases in SIPS (3). This activity is attributed to an increased expression of the lysosomal β-D-galactosidase protein (4). Cellular senescence is also characterized by cell-growth arrest, morphological changes, ROS overproduction (which could explain the maintenance of a senescent state) or gene expression changes.

UVB-induced SIPS represent valuable *in vitro* model to evaluate the efficacy of anti-ageing compounds against the skin ageing processes induced by chronic sun exposure (photoageing).

1,25 dihydroxyvitamin D3 (calcitriol), the active metabolite of vitamin D, is well known for its function in bone metabolism, but cannot be restricted to this. Indeed, its role in photoprotection and immunosupression following UV exposure has been demonstrated in keratinocytes culture (5).

This article was undertaken in order to determine whether 1,25 dihydroxyvitamin D3 is able to help skin cells to repair damages caused by external stresses such as those induced by UV light exposure.

It was shown that the level of appearance of several senescence-associated biomarkers, induced by UVB in human BJ foreskin fibroblasts, strongly decreased by a pre-treatment with calcitriol. The results also support the proof-of-concept that UVB-induced premature senescence can be used in skin photoageing research.

Materials and Methods

1,25 dihydroxyvitamin D3 (calcitriol) was purchased from sigma and used at the concentration of 0,22 µg/ml in 0,05% ethanol.

Abstract

Fighting senescence and the appearance of age signs is of particular interest in a cosmetic point of view. Skin is submitted to intrinsic ageing, linked to genetic characteristics of individuals (chronological ageing) and to extrinsic ageing. Extrinsic ageing is linked to environmental exposure to stress which is mainly represented by UV-exposure. UV are responsible for the appearance of the ageing, by inducing DNA damage, radical oxygen species production, and matrix remodeling.

An *in vitro* model of UV-induced premature senescence of human dermal fibroblasts has been developed, presenting senescence markers, characteristic of photo-ageing. Repeated short non toxic UV-exposures allow cells to reach a senescent phenotype, closely related to replicative ageing. This model has been recognized to evaluate and demonstrate anti-senescence activities of cosmetic ingredients.

This article describes a clear protection after pre-treatment of cells with the active metabolite of vitamine D3, the 1,25 dihydroxyvitamin-D3 (calcitriol) against UV-induced premature senescence. Using gene expression arrays, some interesting insights and pathways are also identified that may be involved in the protective effect.

Olivem® 900 SP

SPF enhancer

OVERALL SUNCARE™

Olivem® 900 SP

is a new multifunctional lipid designed for sunscreen products.

Thanks to its molecular structure, Olivem®900 SP possesses wetting and dispersing properties: when incorporated into a sunscreen product it is able to improve the dispersion of the UV attenuating agents. The improved dispersion of the micro granules of the inorganic UV attenuating agents from the effective wetting activity leads to a better homogeneity. The homogeneous dispersion of the physical filters over the skin provides more complete protection for the skin against the aggression of UV-rays.

Moreover, effective UV protection system can be achieved with even and consistent distribution of the UV attenuating agents over the skin surface: thanks to its dermo-compatibility, the deposition of Olivem®900 SP on the surface of the skin leads to the formation of a physiological and homogeneous film entrapping and carrying the UV-filter particles.

Therefore the addition of Olivem®900 SP **enhances the efficacy of UV-filters** in the formulation thereby **improving protection against UV-rays.**

Patented by B&T – Made in Italy

WWW.OLIVEM900SP.COM

B&T COMPANY
creative users only

Cell culture, exposure to UVB and detection of SA-β-galactosidase

BJ foreskin HDF (ATCC) at early cumulative population doublings were cultured in DMEM (Invitrogen, UK) containing 1% fetal calf serum (FCS, Invitrogen, UK). BJ HDFs at 50-60% of confluence were submitted to four repeated subcytotoxic exposures to UVB at 125 mJ/cm² with the BIOSUN (Vilber Lourmat), with one stress per day, for 4 days, as described in **Fig. 1**.

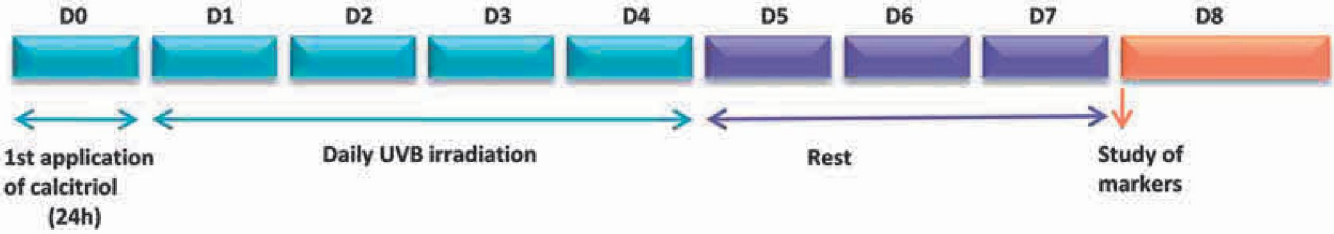

Fig. 1 Schematical procedure of UVB exposure and recovery period (SIPS-UVB model) of human dermal fibroblasts

Untreated control cultures followed the same schedule of medium changes without UVB treatment. For a 24h pre-incubation period and following every stress, the culture medium was replaced by medium containing 0,22 µg/ml calcitriol. After the last UVB exposure, cells were incubated in the presence of calcitriol for 72 hours. At the end of the recovery time, the SA-β-gal activity of the cells was assessed by colorimetry (4). A phosphate-buffered saline solution was used to cover cells during the UVB exposures, in order to discard any filtering effect from the molecule.

Detection of DNA damage (pyrimidine dimers formation)

Cyclobutane pyrimidine dimers (CPDs) were detected by immunofluorescence using the TDM-2 monoclonal antibody, according to the manufacturer's instructions (Medical and Biological Laboratories Ltd, Japan), after the 72h of recovery period.

Cell proliferation assay

After the recovery period, cell proliferation was assessed by BrdU incorporation. BJ HDFs were seeded in a 96-well plate. BrdU Proliferation measurement was performed using the Cell Proliferation ELISA, BrdU (Roche). This colorimetric immunoassay is designed for the quantification of cell proliferation, based on the measurement of BrdU incorporation during DNA synthesis. Measurement of absorbance allows the assessment of proliferation relative to controls.
The effect on cell proliferation was also assessed on fibroblasts and keratinocytes (Lonza, Belgium), without UV exposure. In brief, cells were seeded in 96-well plate and calcitriol was applied for 24h and 72h on cell culture. BrdU proliferation assay was then performed. The controls for these experiments were mitomycin (0,5 ng/ml), bFGF (5 ng/ml) and KGF (20 ng/ml).

ROS production

The oxidant-sensitive probe H2DCFDA (2', 7'-dichlorodihydrofluorescein diacetate, Molecular Probes) was used to determine the intracellular levels of ROS in cells.
72 hours after the last stress, cells were seeded in 24-well plates containing 1 ml of DMEM 10% FCS. The day after, cells were washed with PBS and incubated for 1 hour in the same buffer containing 5 µM H2DCFDA dissolved in ethanol. After washing, the fluorescence intensity was measured using a micro-plate fluorescence reader.

Gene expression study using the »DualChip Human Ageing«

The DualChip™ Human Aging has been designed to efficiently target stress and aging-related gene expression patterns comprising apoptosis, growth factors, extracellular matrix, DNA damage, oxidative stress, cell signaling, cell proliferation, DNA repair, transcription, apoptosis and inflammation (Eppendorf, Germany). The array contains 240 genes, including housekeeping genes and internal controls of the entire process (6).
Total RNA was extracted with the RNeasy Mini Kit (Qiagen) and its concentration was determined by spectrophotometry. Reverse Transcription was performed with a biotin-dNTP mixture. cDNA was then hybridized on the DualChip Human Aging. After staining, the array was scanned using the Eppendorf SilverQuant scanner and following SilverQuant software recommendations. The SilverQuant Analysis software is then used for spot finding and quantification. Array images were further quantified and normalized.

Results

Calcitriol protects human BJ foreskin fibroblasts against senescence-associated beta-glactosidase activity

To determine whether 1,25 dihydroxy vitamin D3 protects against UVB-induced senescence, BJ HDFs were treated by repeated UVB exposures, leading to premature ageing. 0,22 µg/ml calcitriol was included in the culture medium, 24 hours before the stress, following every stress and during the 72-hours recovery phase. At the end of the recovery time, the percentage of SA-β-gal positive cells was measured by colorimetry. As illustrated in **Fig. 2**, the percentage of cells expressing SA β-gal in HDFs after repeated UV exposures was dramatically reduced in the presence of calcitriol, reaching a value even lower to that of the non-irradiated controls.

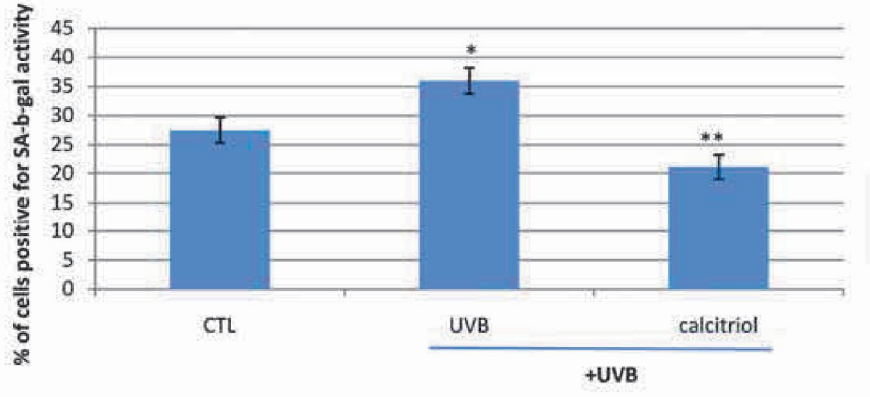

Fig. 2 Percentage of SA-β-galactosidase positive cells after induction of SIPS by repeated UVB exposure. After staining as described (4), 400 cells were counted per well and the number of positive cells for SA-β-gal staining was evaluated. Triplicates were performed

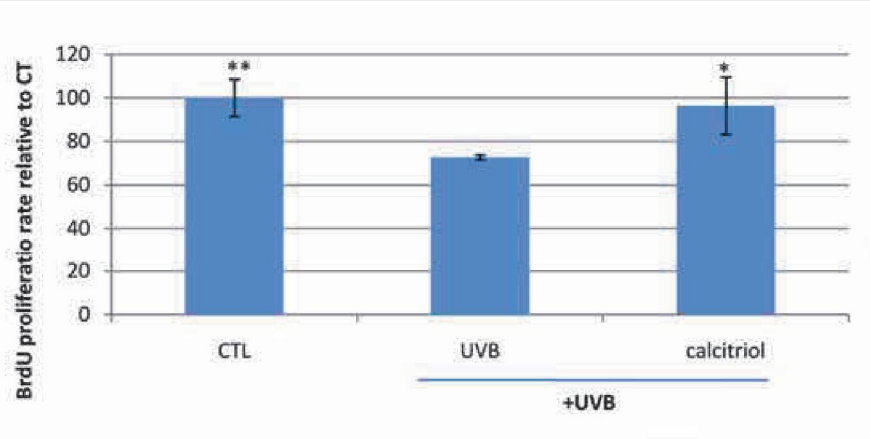

Fig. 3a Cell proliferation rate after 72 h recovery period of the UVB-induced SIPS, evaluated by BrdU incorporation. Results are reported to the control, arbitrarily set to 100%. Histograms represent the mean of three independent experiments with their relative standard deviation

Effect of calcitriol on skin fibroblasts proliferation

The influence of calcitriol on BJ HDFs proliferation was tested using a BrdU incorporation assay. The results in **Fig. 3** demonstrate that calcitriol significantly increased cellular proliferation as compared to UVB-treated cells, restoring a cell proliferation rate similar to control, unexposed cell.

The direct effect of calcitriol on cell proliferation was also evaluated after application in the culture medium of fibroblasts or primary keratinocytes (data not shown) for 24h or 72h. The results showed in **Fig. 4** demonstrate that cellular proliferation is significantly decreased by calcitriol.

Effect of calcitriol on ROS production

After 72h of recovery after the last UV exposure, ROS production was measured by the fluorescent probe H2DCFDA. Results showed in **Fig. 5** indicate a strong decrease of ROS production when cells are treated with calcitriol.

Effect of calcitriol on DNA damage repair after UVB exposure

We tested whether calcitriol could prevent the appearence of UVB-induced senescence by protecting the cells against DNA damages. When the cells were pre-incubated with calcitriol, 24h before UVB exposure and after each exposure, immunofluoresence labeling of CPDs showed that calcitriol seems to decrease the appearance of CPD, as compared to UVB treated cells, without calcitriol **(Fig. 6)**.

Fig. 3b Pictures taken at the end of the recovery period of UVB-induced-SIPS. Cell morphology and number of control (CT) and cells submitted to UVB exposure pretreated with calcitriol are similar, while in the UVB exposed cells condition, cell number is decreased. Arrows indicates senescent cells, flattened and enlarged

Gene expression analysis

Using the low-density array »DualChip™ Human Aging«, the influence of calcitriol on gene expression after induction of SIPS-UVB was studied. Results are presented in Table 1.

Conclusion

SIPS, or cellular senescence induced prematurely by exposure to sub-cytotoxic H_2O_2 or repeated UVB, is characterized by the appearance of several biomarkers, common to the replicative senescence. Among others, cell cycle arrest is observed as well as SA-β-gal activity or the constant overproduction of reactive oxygen species (ROS). The effect of calcitriol was studied in UVB-induced SIPS of human dermal fibroblasts, on the appearance of these biomarkers, after its application before the first UVB exposure, after each exposure and during the recovery period.

Calcitriol, the active metabolite of vitamine D_3 is already known for its photoprotective role *in vitro* on human keratinocytes (7). It was shown that calcitriol has also an effect on the protection against appearance of senescence induced by UVB exposure of human dermal fibroblasts. Indeed, it prevents the appearance of SA-β-gal activity, a well-known marker of senescence.

Senescent cells are also characterized by cell growth arrest. The effect of calcitriol on cell proliferation is very interesting. Indeed, without stress, application of calcitriol induced cell growth arrest, while when added during SIPS, it prevents the cell proliferation arrest. This could probably be explained by a global effect on senescence prevention. Calcitriol is known to act in a biphasic way on keratinocytes proliferation *in vitro*. Indeed, at high concentration, it decreases proliferation, while, at a lower concentration, it increases proliferation (8). Moreover, the results obtained demonstrate that calcitriol doesn't prevent cellular senescence by increasing cell proliferation of non-senescent cells.

Calcitriol also prevents the endogenous overproduction of ROS, which is suspected to be responsible of the maintenance of the senescent state. Indeed, when dermal fibroblasts are submitted to stress, such as UV exposure, ROS are overproduced. The major intracellular ROS intermediate is hydrogen peroxide. It was shown that calcitriol treatments prevented this overproduction, protecting cells against further damage and sustained endogenous metabolic stress.

A preliminary experiment showed its protective effect against the appearance of specific UV-induced CPDs, confirming results obtained by (9) that demonstrate that pre-treatment of keratinocytes with calcitriol prevents DNA damage induced by UV exposure. These preliminary results showed that calcitriol prevents appear-

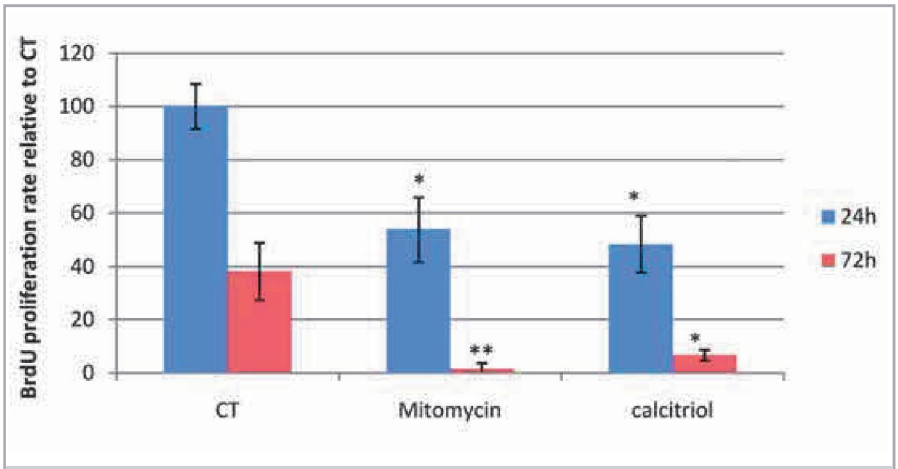

Fig. 4 Fibroblasts proliferation rate evaluated by BrdU incorporation. Results are reported to the control, arbitrarily set to 100%

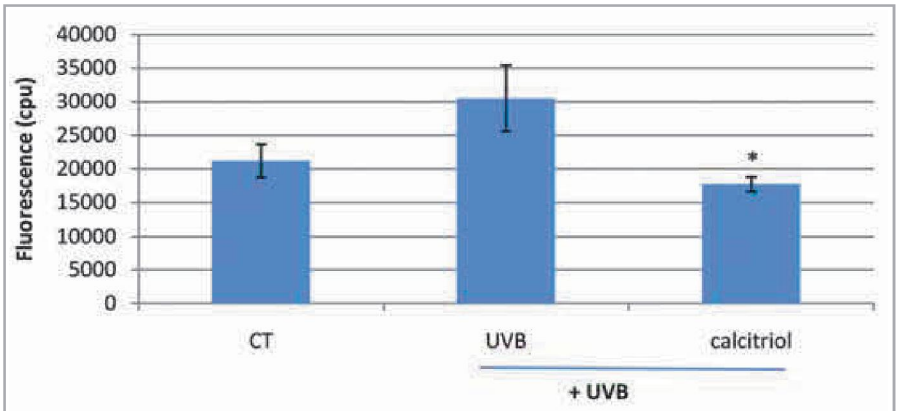

Fig. 5 ROS production evaluated by fluorescence (DCFDA) detection after UVB induced SIPS. Histograms present the results of triplicates

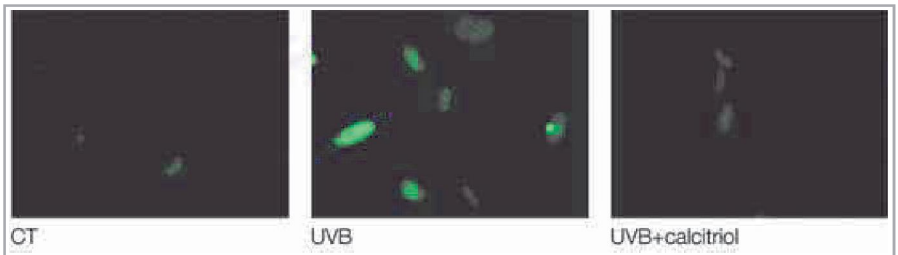

Fig. 6 Pictures taken at the end of the recovery period of UVB-induced-SIPS. Immunofluorescence labeling of CPDs was performed

Table 1 Results of gene expression changes from hybridization on Dual Chip Human aging low density array. Results are expressed as increase or decrease to control untreated test

Gene name	Gene ID	General Function	Norm. Ratio
Enhancer of polycomb homolog 2 (Drosophila)	EPC2	/	1,5145
Amyloid beta (A4) precursor protein-binding, family B, member 1 (Fe65)	APBB1	Alzheimer's disease	1,5552
Prostaglandin-endoperoxide synthase 1 (prostaglandin G/H synthase and cyclooxygenase)	PTGS1	Arachidonic acid metabolism	3,0992
Keratin 19	KRT19	Cell Communication	-1,5047
Collagen, type III, alpha 1 (Ehlers-Danlos syndrome type IV, autosomal dominant)	COL3A1	Cell Communication / Focal adhesion / ECM-receptor interaction	2,0493
Collagen, type VI, alpha 2	COL6A2	Cell Communication / Focal adhesion / ECM-receptor interaction	1,838
Thrombospondin 1	THBS1	Cell Communication / TGF-beta signaling pathway / Focal adhesion / ECM-receptor interaction	-1,9019
Cyclin B1	CCNB1	Cell cycle	2,6879
Cyclin-dependent kinase inhibitor 1B (p27, Kip1)	CDKN1B	Cell cycle	1,6738
E2F transcription factor 1	E2F1	Cell cycle	2,1744
Proliferating cell nuclear antigen	PCNA	Cell cycle	1,6485
Polo-like kinase 1 (Drosophila)	PLK1	Cell cycle	4,2469
Retinoblastoma 1 (including osteosarcoma)	RB1	Cell cycle	1,7762
Cyclin-dependent kinase 4	CDK4	Cell cycle / Tight junction / T cell receptor signaling pathway	1,5125
Collagen, type XV, alpha 1	COL15A1	Cell differentiation	1,4975

Gene name	Gene ID	General Function	Norm. Ratio
Histone 1, H4i	HIST1H4I	Chromosomal processing	2,4634
Kinesin family member 23	KIF23	Chromosomal processing	3,0136
Nuclear receptor subfamily1, group D, member 1	NR1D1	Circadian rhythm	1,5811
Plasminogen activator tissue	PLAT	Complement and coagulation cascades	1,7446
Plasminogen activator,Urokinase	PLAU	Complement and coagulation cascades	1,5233
Tumor necrosis factor receptor superfamily, member 11b (osteoprotegerin)	TNFRSF11B	Cytokine-cytokine receptor interaction	-1,9444
Interleukin 11	IL11	Cytokine-cytokine receptor interaction / Jak-STAT signaling pathway / Hematopoietic cell lineage	2,0776
Poly (ADP-ribose) polymerase family, member 1	PARP1	DNA repair / synthesis	1,6376
Topoisomerase (DNA) II alpha 170kDa	TOP2A	DNA repair / synthesis	2,5339
Ubiquitin-conjugating enzyme E2 variant1	UBE2V1	DNA repair / synthesis	-1,4969
Matrix metallopeptidase 11 (stromelysin 3)	MMP11	Extracellular matrix	-2,5255
Matrix metallopeptidase 3 (stromelysin 1, progelatinase)	MMP3	Extracellular matrix	-4,7008
Insulin like growth factor 1 receptor	IGF1R	Focal adhesion / Adherens junction	1,6364
Insulin-like growth factor binding protein 2, 36kDa	IGFBP2	Growth factor and cytokines	1,6755
V-raf-1 murine leukemia viral oncogene homolog 1	RAF1	MAPK signaling pathway / Dorso-ventral axis formation / Focal adhesion / Gap junction / Natural killer cell mediated cytotoxicity / Long-term potentiation / Long-term depression / Regulation of actin cytoskeleton / Insulin signaling pathway	1,5035
Dihydrofolate reductase	DHFR	One carbon pool by folate / Folate biosynthesis	2,2105
Glucose-6-phosphate dehydrogenase	G6PD	Pentose phosphate pathway / Glutathione metabolism	1,6668
Heme oxygenase (decycling) 1	HMOX1	Porphyrin and chlorophyll metabolism	1,8179
Proteasome (prosome, macropain) 26S subunit, non-ATPase, 11	PSMD11	Proteasome	1,5185
Proteasome (prosome, macropain) 26S subunit, non-ATPase, 12	PSMD12	Proteasome	1,5631
Eukaryotic translation initiation factor 3, subunit 6 48kDa	EIF3S6	Protein biosynthesis	1,5971
HLA-B associated transcript 1	BAT1	Protein metabolism	1,5512
HtrA serine peptidase 1	HTRA1	Proteolysis	2,6521
Ribonucleotide-reductase M1 polypeptide	RRM1	Purine metabolism / Pyrimidine metabolism	2,0955
Polymerase (DNA directed), alpha 2 (70kD subunit)	POLA2	Purine metabolism / Pyrimidine metabolism / DNA polymerase	3,2895
Thymidine kinase 1, soluble	TK1	Pyrimidine metabolism	1,709
Stomatin (EPB72)-like 2	STOML2	Receptor binding	1,7596
Sorcin	SRI	Signal transduction	1,5427
Inhibitor of DNA binding 2, dominant negative helix-loop-helix protein	ID2	TGF-beta signaling pathway	3,6731
Early growth response 1	EGR1	Transcription factor activity	1,994
V-myb myeloblastosis viral oncogene homolog (avian)-like 2	MYBL2	Transcription factor activity	2,3506
Fibromodulin	FMOD	Transforming growth factor beta receptor complex assembly	2,101
Ubiquitin-conjugating enzyme E2C	UBE2C	Ubiquitin mediated proteolysis	2,0697

ISPE certifies the sun protection you're actually providing.

Are you planning to launch a new sunscreen product? In the field of Sun Protection Measurement, our laboratory is considered one of the most experienced and trustworthy in the world. We perform UVA-UVB tests for hundreds of local and global clients, on volunteers or in-vitro, in accordance with the most recent international methods.
Our equipment includes spas for the assessment of water resistance.

ISPE performs a wide range of other safety, efficacy, sensory and consumer tests.
Global expertise, quick service, state-of-the-art methods, clear and reliable reports make ISPE the ideal partner for current and future market leaders worldwide.
ISPE, certified ISO 9001:2000, is a registered member of the Order of Research Laboratories of the Ministry of Scientific Research.

Don't take a risk if you can bet on a certainty.

ISPE Institute of Skin and Product Evaluation

Your ideal partner for guaranteed and certified quality of evaluations

ISPE s.r.l. - dr. Luigi Rigano - via Bruschetti, 1 - 20125 Milan - Italy - Phone +39.02.67.100.695 - E-mail: info@ispe.it - www.ispe.it

International agencies: CQS (Spain) Ph. +34.93.424.0039 - ✉ sistocqs@eic.ictnet.es
DICHEM CHEMICALS (Greece) Ph. +30.210.240.3448 - ✉ info@dichemchemicals.gr • SUMIQUIM (Colombia) Ph. +57.2.551.1776 - ✉ acantillo@sumiquim.com

ance of CPDs, in a UVB-induced senescence model of dermal fibroblast, confirming its photoprotective action.
The use of a specific, stress and ageing-focused low-density DNA array allowed us to identify potential mechanistic pathways of protection, such as the activation of the proteasome, the surexpression of genes involved in anti-oxidant mechanism or the decrease of metalloprotease synthesis, as well as increase of collagen expression.
These results indicate that calcitriol could be considered *in vitro* as a photo-protective agent acting against cellular senescence. Use of safe mimetic vitamin D3 molecules in cosmetic ingredients could be considered as promising approach to slow the appearance of senescence as a result of photo and chronological ageing.

References

(1) *Gilchrest, B.A.* (1980) Prior chronic sun exposure decreases the lifespan of human skin fibroblasts *in vitro*. J Gerontol. 35: 537-541

(2) *Toussaint, O., Remacle, J., Dierick, J.F., Pascal, T., Frippiat, C., Zdanov, S., Magalhaes, J.P., Royer, V.,and Chainiaux, F.* (2002) From the Hayflick mosaic to the mosaics of ageing. Role of stress-induced premature senescence in human ageing. Int J Biochem Cell Biol 34: 1415-1429

(3) *Dimri, G., Lee, X., Basile, G., Acosta, M., Scott, G., Roskelley, C., Medrano, E., Linskens, M., Rubelj, I., Pereira-Smith, O., Peacocke, M.* and *Campisi, J.* (1995) A biomarker that identifies senescent human cells in culture and in aging skin *in vivo*. J. Proc.Natl.Acad.Sci.USA 92: 9363-9367

(4) *Lee, B.Y., Han, J.A., Im, J.S., Morrone, A., Johung, K., Goodwin, E.C., Kleijer, W.J., DiMaio, D., Hwang, E.S.* (2006) Senescence-associated β-galactosidase is lysosomal β-galactosidase. Aging Cell. (2):187-95

(5) *Kuritzky, L.A., Finlay-Jones, J.J., Hart, P.H.* (2008) The controversial role of vitamin D in the skin: immunosuppression vs. photoprotection. Clin Exp Dermatol. 33(2):167-70. Epub 2008 Jan 16. Review

(6) *Borlon, C., Debacq-Chainiaux, F., Hinrichs, C., Scharffetter-Kochanek, K., Toussaint, O., Wlaschek, M.* (2007) The gene expression profile of psoralen plus UVA-induced premature senescence in skin fibroblasts resembles a combined DNA-damage and stress-induced cellular senescence response phenotype.Exp Gerontol. Sep; 42(9):911-23. Epub 2007 Apr 29

(7) *Gupta, R., Dixon, K.M., Shivashni, S.D., Holliday C.J., Slater, M., Halliday, G.M., Reeve V.E., Mason, R.S.* (2007) Photoprotection by 1,25 dihydroxyvitamin D3 is associated with an increase in p53 and a decrease in nitric oxide products. J Invest Dermatol 127, 707-715

(8) *Bollag, W.B., Ducote, J., Harmon, C.S.* (1995). Biphasic effect of 1,25 dihydroxyvitamin D3 on primary mouse epidermal keratinocyte proliferation. J Cell Physiol. 163: 248-256

(9) *Wong, G., Gupta, R., Dixon, K.M., Deo, S.S., Choong, S.M., Halliday, G.M., Bishop, J.E., Ishizuka, S., Norman, A.W., Posner, G.H., Mason, R.S.* (2004) 1,25-Dihydroxyvitamin D and three low-calcemic analogs decrease UV-induced DNA damage via the rapid response pathway. J Steroid Biochem Mol Biol. 89-90(1-5):567-70

(10) *Itin, P.H., Pittelkow, M.R., Kumar, R.* (1994) Effects of vitamin D metabolites on proliferation and differentiation of cultured human epidermal keratinocytes grown in serum-free or defined culture medium. Endocrinology 135:1793-1798

Correspondence address:
* Dr. Nathalie Belot
Coralie Bastin
Mélanie Leroy
Rafaela Fandango
Laurent Naa
Dr. Aline Chretien
Dr. Olivier Toussaint
Dr. Alain Meybeck
Dr. Michel Salmon

StratiCELL SA
Crealys Science Park
Rue Jean Sonet 10
5032 Gembloux
Belgium
Email: nbelot@straticell.com