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Research article

Full title:

Unraveling the interplay between senescent dermal fibroblasts and cutaneous squamous cell carcinoma cell lines at different stages of tumorigenesis

Short title:

Interplay between senescent dermal fibroblasts and cSCC cell lines

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Abstract

Cutaneous Squamous Cell Carcinoma (cSCC) is the second most common type of non-melanoma skin cancer in white-skinned populations. cSCC is associated with sun exposure and aging, which is concomitant with an accumulation of senescent cells in the skin. The involvement of senescent cells in carcinogenesis has been highlighted in several cancer types and an interaction between cSCC cells and senescent cells is proposed, but still little explored. Tumor-associated effects are mostly attributed to the senescence-associated secretory phenotype (SASP). Here, we compared two *in vitro* models of senescence, namely replicative senescence and UVB-stress induced premature senescence (UVB-SIPS), in human dermal fibroblasts and screened for expression of SASP-related genes in our models. Next, ~~we analyzed~~ the impact of senescent fibroblasts on three cSCC isogenic cell lines, representing different stages of keratinocyte malignant transformation, was studied. Only a limited impact on cSCC cell lines' growth and migration has been detected with conditioned media collected from senescent fibroblasts and indirect co-cultures. We then investigated the opposite interaction and found that cSCC cell lines maintained in indirect co-cultures with fibroblasts induced and reinforced their senescence state as shown by an increased proportion of cells positive for the senescence-associated β -galactosidase activity and an increased expression of several SASP-related genes. Moreover, these effects were modulated according to the stage of tumorigenesis of the different cSCC cell lines used. Understanding the interplay between tumor cells and their microenvironment may have important influences in cancer research and therapeutic strategies.

Keywords

- Senescence
- Skin
- Cutaneous squamous cell carcinoma
- Stress-induced premature senescence, SIPS
- UVB
- Senescence-associated secretory phenotype, SASP

Abbreviations

BME: Basal Medium Eagle; CCL2: C-C motif chemokine ligand 2; cSCC: cutaneous Squamous Cell Carcinoma; CTGF: connective tissue growth factor; CXCL1: C-X-C motif chemokine ligand 1; DMEM: Dulbecco's modified Eagle's medium; EGF: epidermal growth factor; FCS: Fetal Calf Serum; FGF2: fibroblast growth factor 2; GAPDH: glyceraldehyde-3-phosphate dehydrogenase; HDF: human diploid fibroblasts; ICAM1: intercellular adhesion molecule 1; IGFBP3: insulin like growth factor binding protein 3; I κ B α : nuclear factor-kappa B inhibitor alpha; IL: interleukin; MMP: matrix metalloproteinase; NF- κ B: nuclear factor-kappa B; RS: replicative senescence; SA- β gal: senescence-associated β -galactosidase; SASP: senescence-associated secretory phenotype; SIPS: stress-induced premature senescence; TGFB1: transforming growth factor beta 1; TLDA: TaqMan Low-Density Array; TNF- α : tumor necrosis factor-alpha; UVR: ultraviolet radiation. μ

1 Introduction

Non-melanoma skin cancers, including basal cell carcinoma (BCC) and cutaneous squamous cell carcinoma (cSCC), are the most common cancers in white-skinned populations { ADDIN EN.CITE <EndNote><Cite><Author>Leiter</Author><Year>2014</Year><RecNum>10</RecNum><DisplayText>(Leiter et al., 2014)</DisplayText><record><rec-number>10</rec-number><foreign-keys><key app="EN" db-id="e0vfs2zwqpxrpcewxt4p5ae4favdv5dxd5wp" timestamp="1499184352">10</key></foreign-keys><ref-type name="Journal Article">17</ref-type><contributors><authors><author>Leiter, U.</author><author>Eigentler, T.</author><author>Garbe, C.</author></authors></contributors><titles><title>Epidemiology of skin cancer</title><secondary-title>Adv Exp Med Biol</secondary-title></titles><periodical><full-title>Adv Exp Med Biol</full-title></periodical><pages>120-40</pages><volume>810</volume><keywords><keyword>Carcinoma, Basal Cell/*epidemiology/metabolism/pathology</keyword><keyword>Carcinoma, Squamous Cell/*epidemiology/metabolism/pathology</keyword><keyword>Europe/epidemiology</keyword><keyword>Female</keyword><keyword>Humans</keyword><keyword>Incidence</keyword><keyword>Male</keyword><keyword>Melanoma/*epidemiology/metabolism/pathology</keyword><keyword>Neoplasms, Radiation-Induced/*epidemiology/metabolism/pathology</keyword><keyword>Risk</keyword><keyword>Skin/metabolism/pathology/radiation effects</keyword><keyword>Skin Neoplasms/*epidemiology/metabolism/pathology</keyword><keyword>Skin Pigmentation/radiation effects</keyword><keyword>Ultraviolet

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steeply worldwide { ADDIN EN.CITE
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2133.2012.10830.x</electronic-resource-num></record></Cite></EndNote> } .

Therefore, identifying etiologic factors and understanding the underlying mechanisms represent a rising challenge for health care services.

cSCC is associated with aging with an average age of 70 years old at diagnosis, and 80 % of cSCC occurring in people aged over 60 years old { ADDIN EN.CITE

<EndNote><Cite><Author>Leiter</Author><Year>2014</Year><RecNum>10</RecN

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urls><url>https://www.ncbi.nlm.nih.gov/pubmed/25207363</url></related-
urls></urls></record></Cite></EndNote>}. The main causal factor associated with

development of cSCC is lifelong cumulative sunlight exposure. Interestingly, skin
aging is in large part 'photoaging' and is characterized by an accumulation of
senescent cells in the skin { ADDIN EN.CITE { ADDIN EN.CITE.DATA }}, raising the
question of whether this accumulation of senescent cells and the appearance of
cSCC with advancing age are in someway linked.

The involvement of senescent cells in carcinogenesis has been highlighted in
several cancer types, such as breast and prostate cancers { ADDIN EN.CITE
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The
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urls></urls><electronic-resource-num>10.1016/j.it.2015.02.009</electronic-resource-

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invasion through induction of epithelial-to-mesenchymal transition (EMT) { ADDIN
EN.CITE { ADDIN EN.CITE.DATA }}. These effects are mostly attributed to the
senescence-associated secretory phenotype (SASP), which is composed of growth
factors, cytokines, chemokines and proteases { ADDIN EN.CITE { ADDIN
EN.CITE.DATA }}. An impact of senescent cells has also been described in skin
cancers. Senescent fibroblasts have been shown to enhance the early stages of skin
carcinogenesis in post-senescence emergent keratinocytes through the involvement

of the MMP/PAR-1 (matrix metalloproteinase/paracrine-activated receptor-1) axis { ADDIN EN.CITE { ADDIN EN.CITE.DATA } } . The involvement of senescent fibroblasts is also described in melanoma initiation and metastasis via the secretion of soluble factors { ADDIN EN.CITE { ADDIN EN.CITE.DATA } } . Senescent dermal fibroblasts also release chemerin, which promotes cSCC migration { ADDIN EN.CITE

<EndNote><Cite><Author>Farsam</Author><Year>2016</Year><RecNum>5</RecNum><DisplayText>(Farsam et al., 2016)</DisplayText><record><rec-number>5</rec-number><foreign-keys><key app="EN" db-id="e0vfs2zwqpxrpcewxt4p5ae4favdv5dxd5wp" timestamp="1495186962">5</key></foreign-keys><ref-type name="Journal Article">17</ref-type><contributors><authors><author>Farsam, V.</author><author>Basu, A.</author><author>Gatzka, M.</author><author>Treiber, N.</author><author>Schneider, L. A.</author><author>Mulaw, M. A.</author><author>Lucas, T.</author><author>Kochanek, S.</author><author>Dummer, R.</author><author>Levesque, M. P.</author><author>Wlaschek, M.</author><author>Scharffetter-Kochanek, K.</author></authors></contributors><auth-address>Department of Dermatology and Allergic Diseases, University of Ulm, Germany.
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Department of Gene Therapy, University of Ulm, Germany.
Department of Dermatology, University Hospital Zurich, Switzerland.</auth-address><titles><title>Senescent fibroblast-derived Chemerin promotes squamous cell carcinoma migration</title><secondary-title>Oncotarget</secondary-title></titles><periodical><full-title>Oncotarget</full-title></periodical><pages>83554-83569</pages><volume>7</volume><number>50</number><keywords><keyword>

Chemerin</keyword><keyword>chemokine CC-motif receptor-like 2
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num></record></Cite></EndNote>}. However, little is known about the crosstalk between senescent fibroblasts and cSCC cells, and particularly about a possible modulation according to their tumorigenesis stage.

Cellular senescence includes replicative senescence, associated with the critical telomere shortening, but also stress-induced premature senescence (SIPS) generated by DNA damage and/or oxidative stress and oncogene-induced senescence (OIS) following oncogene activation { ADDIN EN.CITE { ADDIN EN.CITE.DATA }}. It is worth noting that even if these cells are irreversibly growth-arrested, some other senescence characteristics, such as the SASP, can be modulated according to the senescence inducer { ADDIN EN.CITE
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num>10.1146/annurev-pathol-121808-102144</electronic-resource-
num></record></Cite></EndNote>}. Therefore, senescence studies may inform on
relevant senescence inducers within specific tissue contexts.

With reference to the skin, its ~~Skin~~ aging is strongly influenced by
environmental factors, notably solar radiation and particularly ultraviolet radiation
(UVR) { ADDIN EN.CITE
<EndNote><Cite><Author>Krutmann</Author><Year>2017</Year><RecNum>26</R
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Department of Dermatology, University Hospital Center of Nice, France; INSERM U1065, team 12, C3M, Nice, France.</auth-address><titles><title>The skin aging exposome</title><secondary-title>J Dermatol Sci</secondary-title></titles><periodical><full-title>J Dermatol Sci</full-title></periodical><pages>152-161</pages><volume>85</volume><number>3</number><keywords><keyword>Air pollution</keyword><keyword>Cosmetic strategies</keyword><keyword>Infrared radiation</keyword><keyword>Nutrition</keyword><keyword>Ultraviolet radiation</keyword><keyword>Visible light</keyword></keywords><dates><year>2017</year><pub-dates><date>Mar</date></pub-dates></dates><isbn>1873-569X (Electronic)
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energetic component of UVR to reach the earth's surface and UVB has been shown to induce a senescent phenotype in various skin cell types { ADDIN EN.CITE { ADDIN EN.CITE.DATA }}.

In this study, we used two relevant *in vitro* models of senescence in the context of skin aging and cSCC, which are replicative senescence and UVB-induced senescence (UVB-SIPS, { ADDIN EN.CITE { ADDIN EN.CITE.DATA }}) in dermal HDFs (human diploid fibroblasts). We investigated their SASP and their interaction with cSCC cell lines, using three isogenic cSCC cell lines that represent different stages of malignant transformation with the same genetic background { ADDIN EN.CITE { ADDIN EN.CITE.DATA }}. These stages of squamous epidermal carcinogenesis are carcinoma *in situ*, invasive primary SCC and distant lymph node metastasis. ~~We first analyzed~~ Firstly, the interaction of senescent HDFs on cSCC cell lines was analyzed by using conditioned media and indirect co-cultures. Secondly, we studied the impact of these cSCC cell lines on HDFs by indirect co-cultures. Our results strongly suggest for the first time that cSCC cell lines highly induce and reinforce HDFs senescence, with an increase in senescence-associated β -galactosidase (SA- β gal) activity and in SASP-related genes expression. Interestingly these effects are modulated according to the tumorigenesis stage. Finally, cSCC cell lines-co-cultures are associated with NF- κ B activation in HDFs.

2 Materials and methods

2.1 Cell culture

Dermal HDFs (AG04431 from Coriell Institute for Medical Research) were grown in BME (Basal Medium Eagle, Gibco) supplemented with 10 % FCS (Fetal Calf Serum, Gibco) and 2 mM L-glutamine (Gibco) until replicative senescence or

submitted to UVB stresses to induce UVB-SIPS as previously described { ADDIN EN.CITE { ADDIN EN.CITE.DATA }}. For UVB-SIPS, HDFs were seeded at 8,000 cells per cm² and control cells were kept in the same culture conditions without UVB exposure.

PM1, MET1 and MET4 cell lines were isolated from the same individual and represent different stages of tumorigenesis { ADDIN EN.CITE { ADDIN EN.CITE.DATA }}. PM1 cell line is derived from dysplastic forehead skin and believed to represent carcinoma *in situ* or pre-invasive dysplasia; MET1 cell line is derived from a primary cSCC arising on dorsal hand and MET4 cell line is derived from an axillary lymph node metastasis from MET1. These keratinocytes cell lines were cultivated in DMEM plus Ham's F-12 Nutrient Mix (Gibco, ratio of 3:1) containing 10 % FCS, 400 ng/ml hydrocortisone (Sigma-Aldrich) and 10 ng/ml mouse EGF (AbD Serotec) as described previously { ADDIN EN.CITE { ADDIN EN.CITE.DATA }}. A431 cSCC cell line was purchased from the American Type Cell Culture Collection (ATCC) and was grown in DMEM supplemented with 10 % FCS. All cells were maintained at 37°C in a humidified atmosphere containing 5 % CO₂. Bright-field images of all the cell types used in this study are shown in Fig. S1.

2.2 Conditioned media and co-culture experiments

For conditioned media collection, HDFs were seeded at 5,300 (young) or 8,000 (replicative senescent) cells per cm² in BME with 1 % FCS and 2 mM L-glutamine. The next day, media were changed. For SIPS HDFs, media were changed 64 hours after the last UVB stress. Conditioned media were collected after 48 hours of incubation from HDFs with a similar density (approximately 55,000 cells/ml). The "BME" condition refers to medium incubated with glutamine and FCS

but without fibroblast. After collection, media were centrifuged at 1,000 rpm for 7 min at 4°C to eliminate cell debris and they were used immediately without freezing.

For indirect co-culture experiments, the protocol is illustrated in Fig. 44. 10,000 dermal HDFs per cm² were seeded in 6-well plates (Corning) in BME with 1 % FCS and 2 mM L-glutamine. CTL-SIPS and UVB-SIPS HDFs were seeded at 72 hours after the last UVB stress. The next day (day 0), cSCC cell lines were seeded in cell culture inserts (membrane pore size of 0.4 µm, Corning). PM1, MET1 and MET4 were seeded at 2,100 cells/cm² in DMEM plus Ham's F-12 Nutrient Mix containing 1 % FCS, hydrocortisone and mouse EGF. A431 were plated at 1,500 cells/cm² in DMEM with 1 % FCS. Media were changed at day 3 of co-culture.

2.3 TLDA analysis

Total RNA was isolated using the RNeasy Mini kit or Micro kit and Qiacube (Qiagen). RNA integrity and quality was validated with RNA 6000 Nano kit and 2100 Bioanalyzer (Agilent Technologies) and reverse-transcribed using the High-Capacity cDNA Reverse Transcription kit (Applied Biosystems). A customized TaqMan Low-Density Array (TLDA) allowing studying the expression of 48 SASP-related genes was generated (list of the genes on Table S1). TLDA analyses were performed using TaqMan™ Universal PCR Master Mix no AmpErase UNG and ViiA7 instrument according to the manufacturer's instructions (Applied Biosystems). Relative abundance was determined with the $\Delta\Delta C_q$ method, normalized to the mRNA abundance of *GAPDH* (glyceraldehyde-3-phosphate dehydrogenase) for Table 1 and *GAPDH* and *RNA18S* for Table 2 and expressed as relative to the stated control.

2.4 Cell growth measurement

For conditioned media experiments, 16,000 cells/cm² for PM1 and 8,000 cells/cm² for MET1, MET4 and A431 cell lines were seeded in 12-well plates in their respective medium with 1 % FCS and incubated the next day with conditioned media for 24 hours. For co-culture experiments, cell culture insert membranes were cut off carefully with a blade and put in 6-well plates at day 1, 3 and 6 of co-culture.

Cell growth was measured with the CellTiter 96 AQueous One Solution Cell Proliferation Assay (Promega). Cells were incubated 3 hours with 200 µl of reagent in 1 ml of culture medium at 37°C in a humidified, 5 % CO₂ atmosphere. The absorbance was then measured at 490 nm. To avoid background, we calculated corrected absorbance by subtracting the absorbance of control wells (containing medium and reagent without cells).

2.5 Migration and invasion assays

For migration assays, 30,000 cells/well for PM1, 10,000 cells/well for MET1 and 15,000 cells/well for MET4 and A431 cell lines were seeded onto Falcon cell culture inserts with 8 µm pore size (24-well plates, Corning) in their respective medium with 1 % FCS. For invasion assays, MET1 (10,000 cells/well), MET4 (15,000 cells/well) and A431 (30,000 cells/well) cell lines were seeded onto BioCoat Matrigel Invasion Chambers with 8 µm pore size coated with Matrigel Matrix (24-well plates, Corning) in their respective medium with 1 % FCS following manufacturer's instructions. Conditioned media were incubated in the lower chambers for 24 hours at 37°C in a humidified, 5 % CO₂ atmosphere. Cells on the lower surface of the membrane were rinsed, fixed and stained with 0.2 % crystal violet in 2 % aqueous ethanol (Merck Millipore) for 2 min. After several washes in distilled water, the non-migrating and non-invading cells were removed from the upper surface by twice

scrapings with cotton swabs (Copan). Membranes were photographed with the BX-63 microscope (Olympus, magnification 4 x) and analyzed for cell counting.

2.6 Senescence-associated beta-galactosidase (SA-βgal) activity

At day 1 and 6 of co-culture, HDFs were seeded in 6-well plates (Corning) at a density of 1,000 cells/cm² in DMEM plus Ham's F-12 Nutrient Mix containing 1 % FCS, hydrocortisone and mouse EGF. The next day SA-βgal staining was performed as described in { ADDIN EN.CITE { ADDIN EN.CITE.DATA } } during 16 hours. The proportion of SA-βgal positive cells was determined by counting 400 cells per well.

2.7 RT-qPCR analysis

Total RNA was isolated using the RNeasy Micro kit and Qiacube (Qiagen) and reverse-transcribed using Transcriptor First Strand cDNA Synthesis Kit (Roche) following manufacturer's instructions. qPCR was performed using SYBR Green PCR Master mix (Roche), primers and the StepOnePlus Real-Time PCR system (Applied Biosystems). Primers used were: CCL2-F: CATTGTGGCCAAGGAGATCTG; CCL2-R: AGTGAGTGTTCAAGTCTTCGGAGTT; GAPDH-F: ACCCACTCCTCCACCTTTGAC; GAPDH-R: GTCCACCACCCTGTTGCTGTA; IL1B-F: GCCCTAAACAGATGAAGTGCTC; IL1B-R: GAGATTCGTAGCTGGATGCC; IL8-F: CTGGCCGTGGCTCTCTTG and IL8-R: GGGTGGAAAGGTTTGGAGTATG. Relative abundance was determined with the $\Delta\Delta C_q$ method, normalized to the mRNA abundance of *GAPDH* and expressed relative to the stated control.

2.8 Nuclear p65 staining

HDFs were analyzed at day 6 of co-culture directly in 6-well plates. For positive controls, HDFs were incubated with 20 ng/ml tumor necrosis factor- α (TNF- α R&D Systems) for 30 min in BME with 1 % FCS and 2 mM L-glutamine. Negative control was kept in the same culture conditions without TNF- α incubation.

Cells were fixed with 4 % paraformaldehyde in PBS (Merck Millipore) for 10 min, permeabilized in 1 % Triton X-100 (Sigma-Aldrich) in PBS for 5 min and incubated with rabbit anti-NF- κ B p65 antibody (1:400, Cell Signaling, AB_10859369) diluted in 3 % bovine serum albumin (Santa Cruz Biotechnology) in PBS overnight at 4°C. Cells were then incubated with the secondary antibody Alexa Fluor 488 goat anti-rabbit IgG (1:1,000, Molecular Probes, AB_143165) diluted in 3 % bovine serum albumin in PBS for 1 hour at room temperature. Nuclei were stained with DAPI (1ng/ml in PBS, Sigma-Aldrich) during 15 minutes at room temperature. Cells were kept in PBS in the dark and analyzed with the BD Pathway 855 (BD Biosciences). Segmentation and analyses were performed with BD AttoVision software, which identified nuclei through DAPI staining and quantified the NF- κ B p65 staining intensity in these regions of interest. An average of 450 cells was counted per condition.

2.9 Western blot analysis

Total cell protein lysates were obtained using a buffer containing 40 mM Tris (Merck Millipore), 150 mM KCl (Merck Millipore), 1 mM EDTA (Merck Millipore), pH 7.5, 1 % Triton X-100 (Sigma-Aldrich), supplemented with Complete Protease Inhibitor Cocktail (Roche) and 4 % phosphatase inhibitor buffer (25 mM Na₃VO₄ (Sigma-Aldrich), 250 mM 4-nitrophenyl phosphate (Sigma-Aldrich), 250 mM β -glycerophosphate (VWR), 125 mM NaF (Merck Millipore)). Protein concentration was

determined by Pierce 660 nm Protein Assay (Thermo Scientific). 15 μ g of proteins were separated by SDS-PAGE on 10 % Mini-PROTEAN TGX precast gels (Biorad) and electrotransferred on a polyvinylidene fluoride membrane (Merck-Millipore). Molecular weights were determined with SeeBlue Plus2 pre-stained protein standard (Invitrogen). Membranes were blocked 1 hour at room temperature in Odyssey blocking buffer (LI-COR) and then overnight at 4°C in Odyssey blocking buffer with 0.1 % Tween 20 (Bio-Rad) containing a primary antibody. The primary antibodies used are mouse anti-E-cadherin (1:1,000, Abcam, AB_300946), rabbit anti-vimentin (1:1,000, Cell Signaling, AB_10695459) and mouse anti- α -tubulin (1:10,000, Sigma-Aldrich, T5168). After 4 washes of 5 minutes in PBS with 0.1 % Tween 20, the incubation with the secondary antibody (IRDye 680RD goat anti-mouse IgG, LI-COR, AB_10956588 or 800CW goat anti-rabbit IgG, LI-COR, AB_621843, both 1:10,000) was performed for 1 hour in Odyssey blocking buffer with 0.1 % Tween 20. After 4 washes of 5 minutes in PBS-Tween and 2 washes in PBS, the membrane was dried 1 hour at 37°C and scanned using Odyssey infrared imaging system V3.0 (LI-COR) for the quantification. 8 μ g of proteins were separated by SDS-PAGE on 10 % SDS-PAGE gels and electrotransferred on a polyvinylidene fluoride membrane (Merck Millipore). Molecular weights were determined with color pre-stained protein standard (New England Biolabs). Membranes were blocked 1 hour at room temperature in Odyssey blocking buffer (LI-COR) and then overnight at 4°C in Odyssey blocking buffer with 0.1 % Tween 20 (Bio-Rad) containing a primary antibody. The primary antibodies used are rabbit anti-I κ B α (1:500, Santa Cruz Biotechnology, AB_2235952) and mouse anti- β -actin (1:10,000, Sigma-Aldrich, AB_476744). After 4 washes of 5 minutes in PBS with 0.1 % Tween 20, the incubation with the secondary antibody (IRDye 680RD goat anti-rabbit IgG, LI-COR, AB_621841 or IRDye 680RD goat anti-mouse IgG, LI-COR, AB_10956588, both 1:10,000) was performed for 1 hour in

Odyssey blocking buffer with 0.1 % Tween 20. After 4 washes of 5 minutes in PBS-Tween and 2 washes in PBS, the membrane was dried 1 hour at 37°C and scanned using Image Studio Lite software V3.1.4 (LI-COR) for the quantification.

2.10 Statistical analysis

Experiments were performed at least three times independently except when otherwise noted. Results are expressed as means $\pm$ SEM (standard error of the mean). Statistical analyses were performed using R software (version 3.2.3 from the R Foundation for Statistical Computing).

Two-tailed T-test (Table 1, and: Fig. S6S7 and S10C) or two ways analysis of variance with *post-hoc* Tukey's test were performed. For Fig. 2 and Fig. S2-S5 Fig. 1-3, 5, 10 and Fig. S6, S12, each experiment was treated as a matched set to anticipate experiment-to-experiment variability and was considered as one factor of the ANOVA. Normality and homoscedasticity of the data were assessed with Shapiro-Wilk and Levene's test respectively. When normality or homogeneity of variances could not be assumed (p -value < 0.05), statistical analyses were performed on transformed data (log-transformed data for Table 1, Fig. 2-4 Fig. 1B-C, 3, 5, 7, 8, Table S2 and Fig. S2B-C, S4 and S5 Fig. S6 or square-root-transformed data for Fig. S7 Fig 6). In order to facilitate interpretation, untransformed data are shown. When normality or homoscedasticity tests failed on transformed data (p -value < 0.05), Kruskal-Wallis test followed by Wilcoxon-Mann-Whitney test were performed instead (Fig. 3A, 4A and 4C Fig. 7A, 8A and 8C).

3 Results

3.1 Dermal HDFs in replicative or in UVB-induced senescence display specific SASP-related gene expression changes

To determine the expression of SASP-related genes in HDFs in replicative senescence (RS) or in UVB-SIPS, we designed a customized TLDA (Applied Biosystems) to analyze simultaneously the expression of 48 SASP-related genes (Table S1). HDFs in RS displayed a significant increase in *IL1B* (interleukin 1 beta), *ICAM1* (intercellular adhesion molecule 1), *CTGF* (connective tissue growth factor) and *IL6* relative mRNA abundances when compared with pre-senescent (young) HDFs (Table 1A). They also displayed a significant decrease in several SASP-related gene abundances, such as *CXCL1* (C-X-C motif chemokine ligand 1) and *CXCL2*. HDFs in UVB-SIPS shared three common expression changes with RS, the increased *IL1B*, *IL6* and *CTGF* relative mRNA abundances (Table 1B). UVB-SIPS HDFs also displayed a significant increase in *MMP1*, *MMP3*, *CXCL1*, *CXCL2*, *CCL2* (C-C motif chemokine ligand 2), *IL8*, *IGFBP3* (insulin like growth factor binding protein 2), *IGFBP6*, *FGF2* (fibroblast growth factor 2) and *TGFB1* (transforming growth factor beta 1) relative mRNA abundances when compared with their respective control (CTL-SIPS), not exposed to UVB exposure. These results highlighted that HDFs in RS or in UVB-SIPS are characterized by common but also by specific expression changes for SASP-related genes, which may be reminiscent of differential impacts on their microenvironment.

3.2 Impact of senescent dermal HDFs on cSCC cell lines growth, migration and invasion according to their stage of tumorigenesis

The impact of dermal senescent HDFs on cSCC is poorly described. The aim of our present study was first to see if this impact could be modulated according to the stage of tumorigenesis of this type of cancer. For this purpose, ~~We selected~~ cSCC lines isolated from the same individual and representing different stages of tumorigenesis { ADDIN EN.CITE { ADDIN EN.CITE.DATA }} were selected. PM1 cell line is derived from dysplastic skin and represents early stage of malignant transformation; MET1 cell line is derived from a primary SCC with invasive properties and MET4 cell line is derived from a distant metastasis { ADDIN EN.CITE { ADDIN EN.CITE.DATA }}. These cell lines represent therefore an isogenic skin cancer progression model { ADDIN EN.CITE { ADDIN EN.CITE.DATA }}. We validated that they displayed different expression patterns of EMT markers as described previously (Fig. S42). All cSCC cell lines were subsequently used at low passage numbers, with marked differences for EMT markers.

We first tested the impact of conditioned medium collected after 48 hours of culture from senescent HDFs in RS or in UVB-SIPS and their respective controls (young and CTL-SIPS; see Fig. S3 for bright-field images of HDFs) on the growth, the migration and the invasion of the cSCC cell lines after 24 hours of incubation. ~~Regarding the cSCC cell lines at different stages of tumorigenesis, we did not observe an~~ No significant impact of conditioned medium from HDFs in RS or in UVB-SIPS was observed, except a growth increase for PM1 incubated with UVB-SIPS conditioned medium (~~Fig. S2-S4~~ Fig. 1-3; see Fig. S4 and S5 for bright-field images of migrating and invading cells). Moreover, ~~we have observed~~ a negative and not

negligible impact of these conditioned media was noticed compared to the medium alone.

To the extent that the impact of senescent HDFs on cSCC cell migration (including A431 cell line) ~~cell migration~~, has been shown recently { ADDIN EN.CITE

<EndNote><Cite><Author>Farsam</Author><Year>2016</Year><RecNum>5</Rec

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Chemerin promotes squamous cell carcinoma migration</title><secondary-

title>Oncotarget</secondary-title></titles><periodical><full-title>Oncotarget</full-

title></periodical><pages>83554-

83569</pages><volume>7</volume><number>50</number><keywords><keyword>

Chemerin</keyword><keyword>chemokine CC-motif receptor-like 2

(CCRL2)</keyword><keyword>cutaneous squamous cell carcinoma (cSCC)</keyword><keyword>senescence-associated secretory phenotype (SASP)</keyword><keyword>tumor migration</keyword></keywords><dates><year>2016</year><pub-dates><date>Dec 13</date></pub-dates></dates><isbn>1949-2553 (Electronic)
1949-2553 (Linking)</isbn><accession-num>27907906</accession-num><urls><related-urls><url>https://www.ncbi.nlm.nih.gov/pubmed/27907906</url></related-urls></urls><custom2>PMC5347788</custom2><electronic-resource-num>10.18632/oncotarget.13446</electronic-resource-

num></record></Cite></EndNote> } , ~~we analyzed~~ the impact of our conditioned media on A431 was analyzed (Fig. S56). As expected, replicative senescent HDFs conditioned media promoted A431 cell line migration when compared with young HDFs conditioned media (Fig. S5B6B). However, they did not affect A431 cell line growth or invasion (Fig. S5A6A and S5C6C). Moreover, UVB-SIPS HDFs conditioned media did not show any effect.

~~We then performed~~ Indirect co-culture experiments were then performed by using cell culture inserts. Indirect co-cultures have several advantages among which the population-specific detection of cellular changes and the bidirectional exchange. ~~Co-cultures were performed by seeding~~ Senescent HDFs (in RS or in UVB-SIPS), or not (young or CTL-SIPS), were seeded in the dish, ~~with cultivation of~~ and cultivated with cSCC cell lines at different stages of tumorigenesis in the above inserts (Fig. 14). ~~Indirect co-cultures have several advantages among which the population-specific detection of cellular changes and the bidirectional exchange.~~ We first studied the impact of HDFs' senescence on cancer cell growth. To assess our co-culture protocol, ~~we evaluated~~ the impact of young or replicative senescent WI-38 co-

cultures on MDA-MB-231 growth, which has been described previously { ADDIN EN.CITE

<EndNote><Cite><Author>Krtolica</Author><Year>2001</Year><RecNum>3</RecNum><DisplayText>(Krtolica et al., 2001)</DisplayText><record><rec-number>3</rec-number><foreign-keys><key app="EN" db-id="e0vfs2zwqpxrpcewxt4p5ae4favdv5dxd5wp" timestamp="1494256294">3</key></foreign-keys><ref-type name="Journal Article">17</ref-type><contributors><authors><author>Krtolica, A.</author><author>Parrinello, S.</author><author>Lockett, S.</author><author>Desprez, P. Y.</author><author>Campisi, J.</author></authors></contributors><auth-address>Life Sciences Division, Lawrence Berkeley National Laboratory, Berkeley, CA 94720, USA.</auth-address><titles><title>Senescent fibroblasts promote epithelial cell growth and tumorigenesis: a link between cancer and aging</title><secondary-title>Proc Natl Acad Sci U S A</secondary-title></titles><periodical><full-title>Proc Natl Acad Sci U S A</full-title></periodical><pages>12072-7</pages><volume>98</volume><number>21</number><keywords><keyword>Aging/*physiology</keyword><keyword>Animals</keyword><keyword>Cell Aging/physiology</keyword><keyword>Cell Division</keyword><keyword>Cell Line</keyword><keyword>Epithelial Cells/cytology/physiology</keyword><keyword>Fibroblasts/cytology/*physiology</keyword><keyword>Kinetics</keyword><keyword>Mice</keyword><keyword>Mice, Nude</keyword><keyword>Neoplasms/*etiology</keyword><keyword>Neoplasms, Experimental/etiology/physiopathology</keyword><keyword>Precancerous Conditions</keyword></keywords><dates><year>2001</year><pub-dates><date>Oct 09</date></pub-dates></dates><isbn>0027-8424

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0027-8424 (Linking)</isbn><accession-num>11593017</accession-num><urls><related-urls><url>https://www.ncbi.nlm.nih.gov/pubmed/11593017</url></related-urls></urls><custom2>PMC59769</custom2><electronic-resource-num>10.1073/pnas.211053698</electronic-resource-num></record></Cite></EndNote> } , was evaluated. As expected, replicative senescent WI-38 significantly promoted MDA-MB-231 growth from 5 days of co-culture when compared with young WI-38 (Fig. S6S7).

Co-cultures were then performed with HDFs in RS or in UVB-SIPS, or with their respective controls (young or CTL-SIPS), and cSCC cell lines at different stages of tumorigenesis. We did not detect any difference in growth for PM1, MET1 and MET4 maintained in co-cultures with senescent HDFs, except for PM1 maintained with UVB-SIPS HDFs in ~~UVB-SIPS~~ (at day 1) and for MET4 maintained with RS HDFs in ~~RS~~ (at day 1 and at day 6), ~~but these effects, even if significant, appear to be limited~~ (Fig. 25; see Fig. S8 for bright-field images of cSCC cell lines during co-cultures). However, these effects, even if significant, appear to be limited. These results confirm the ones obtained with the conditioned media. Therefore, ~~and we can conclude that~~ the impact of the senescent HDFs in these three cSCC cell lines seems to be is very limited. This result is surprising but shows that the pro-tumorigenic effect of senescent HDFs is not universal, and that it may depend on the senescence model and on the type of cancer cell.

3.3 cSCC cell lines highly affect dermal HDFs senescence and ~~SASP expression~~

Next, we ~~therefore~~ decided to take an interest in the two-way exchange and to study the impact of cSCC cells on HDFs. Indeed, if the impact of senescent cells on

their microenvironment is highly described, little is known about the other way around effect. We focused this investigation on replicative senescence. Thus, ~~we investigated by using indirect co-cultures,~~ the impact of cSCC cell lines on the SA- β gal activity of young and RS HDFs, at day 1 and day 6 of the co-cultures, was investigated (Fig. S76; see Fig. S9 for bright-field images of SA- β gal activity detection in HDFs). Interestingly, after 6 days of co-culture, a higher proportion of SA- β gal positive cells was detected in young HDFs co-cultured with PM1, MET1 and MET4, when compared with the control condition (young HDFs alone). This effect was more pronounced in MET1- and MET4-co-cultures, when compared to PM1-co-culture. No co-culture effect was observed in replicative senescent HDFs.

3.4 cSCC cell lines highly increase SASP-related gene expression in dermal HDFs

We then investigated the impact of PM1 and MET1-co-cultures on SASP-related gene expression in HDFs (Table 2) by using the same customized TLDA (Table S1). RNA was isolated after 1 or 6 days of co-culture in young and replicative senescent HDFs. This screening showed that senescence (between young and RS HDFs) and/or co-culture (between HDFs alone or co-cultured with PM1 or MET1) modulated several SASP-related gene expression changes after 6 days of co-culture.

For young and replicative senescent HDFs, the relative expression level of four genes (*CCL2*, *CXCL2*, *IL1B* and *MMP1*) was significantly increased with senescence and co-culture (Table 2). Indeed, PM1 and MET1 cell lines-co-cultures induced a significant gene expression increase in young and replicative senescent HDFs, when compared with their respective controls (alone). Only the expression of *TIMP2* (*TIMP metalloproteinase inhibitor 2*) was significantly increased with senescence, but reduced with co-culture (with PM1 cell line). Moreover, an induction

of the mRNA abundance of four others genes (*CCL20*, *CXCL1*, *ICAM1* and *IL8*) was also observed with co-culture. Finally, *FGF2*, *IGFBP3*, *IGFBP5*, *MMP3*, *MMP10* and *TNFRSF11B* gene expressions were upregulated, and *IGFBP2*, *PTGS1* (*prostaglandin-endoperoxide synthase 1*) and *VCAM1* (*vascular cell adhesion molecule 1*) gene expressions were downregulated with senescence.

To confirm these results on a larger sample size, we studied *CCL2*, *IL1B* and *IL8* gene expression in co-cultures of young and replicative senescent HDFs with PM1, MET1 and MET4 cells (Fig. 37; see Fig. S10 for bright-field images of HDFs during co-cultures). The three genes were overexpressed in RS HDFs at day 1 of the co-culture. After 6 days of co-cultures, ~~we observe~~ a significant increase of these three genes in young and in RS HDFs when co-cultured with the three cSCC cell lines was observed. Moreover, for *IL1B* and *IL8* gene expression, the co-culture impact was higher with MET1-co-culture, when compared with MET4-co-culture, showing a similar effect but with an amplitude dependent on the tumorigenesis stage.

~~This effect~~ Thus, co-cultures seems to induce and to reinforce the senescent state of HDFs.

In order to confirm these results with another cSCC cell line isolated from another patient, we performed co-culture experiments with A431 cell line (Fig. 4- Fig. 8; see Fig. S11 for bright-field images of HDFs during co-cultures). As this cell line was highly proliferative, co-culture experiments were stopped at day 3, when cell confluence was reached. We confirmed the overexpression of these three genes in RS HDFs at day 1, and in young and RS HDFs maintained in co-culture with A431 at day 3.

3.5 cSCC cell lines-co-cultures are associated with NF-κB activation in dermal HDFs

As these cSCC cell lines induced an increase in SASP-related gene expressions in HDFs, we can wonder if regulatory networks of the SASP are activated. NF-κB (nuclear factor-kappa B) is one master regulator of most SASP-related gene expressions { ADDIN EN.CITE <EndNote><Cite><Author>Malaquin</Author><Year>2016</Year><RecNum>4</RecNum><DisplayText>(Malaquin et al., 2016)</DisplayText><record><rec-number>4</rec-number><foreign-keys><key app="EN" db-id="e0vfs2zwqpxrpcewxt4p5ae4favdv5dxd5wp" timestamp="1494410660">4</key></foreign-keys><ref-type name="Journal Article">17</ref-type><contributors><authors><author>Malaquin, N.</author><author>Martinez, A.</author><author>Rodier, F.</author></authors></contributors><auth-address>CRCHUM et Institut du cancer de Montreal, Montreal, QC, Canada.
CRCHUM et Institut du cancer de Montreal, Montreal, QC, Canada; Universite de Montreal, Departement de radiologie, radio-oncologie et medecine nucleaire, Montreal, QC, Canada. Electronic address: rodierf@mac.com.</auth-address><titles><title>Keeping the senescence secretome under control: Molecular reins on the senescence-associated secretory phenotype</title><secondary-title>Exp Gerontol</secondary-title></titles><periodical><full-title>Exp Gerontol</full-title></periodical><pages>39-49</pages><volume>82</volume><keywords><keyword>Epigenetics</keyword><keyword>Intracellular signalling pathways</keyword><keyword>Nuclear factor kappa B</keyword><keyword>Senescence-messaging secretome</keyword><keyword>Signal transduction</keyword><keyword>Signalling cascade</keyword></keywords><dates><year>2016</year><pub-

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urls></urls><electronic-resource-num>10.1016/j.exger.2016.05.010</electronic-

resource-num></record></Cite></EndNote>}. As p65 is an essential subunit of the

NF- κ B complex, we investigated the nuclear p65 staining intensity in HDFs alone or

co-cultured with cSCC cell lines in order to highlight a possible activation (Fig. S89).

TNF- α treated HDFs provided a positive control for p65 nuclear activation (Fig.

S8A9A).

The fluorescence intensity of nuclear p65 per cell was significantly increased in RS HDFs when compared with young HDFs (Fig. S8B and S8C 9B and 9C), as

expected

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rodierf@mac.com.</auth-address><titles><title>Keeping the senescence secretome

under control: Molecular reins on the senescence-associated secretory

phenotype</title><secondary-title>Exp Gerontol</secondary-title></titles><periodical><full-title>Exp Gerontol</full-title></periodical><pages>39-49</pages><volume>82</volume><keywords><keyword>Epigenetics</keyword><keyword>Intracellular signalling pathways</keyword><keyword>Nuclear factor kappa B</keyword><keyword>Senescence-messaging secretome</keyword><keyword>Signal transduction</keyword><keyword>Signalling cascade</keyword></keywords><dates><year>2016</year><pub-dates><date>Sep</date></pub-dates></dates><isbn>1873-6815 (Electronic)
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resource-num></record></Cite></EndNote>}. Moreover after 6 days of co-culture, there was a significant impact of PM1 and MET4-co-cultures on young and RS HDFs' staining when compared with the control conditions alone. This is consistent with our previous results that showed that cSCC cell lines-co-cultures promoted SASP-related gene expressions. Surprisingly there was no significant impact of MET1-co-cultures when compared with the control conditions alone on nuclear p65 intensity staining. Finally, this staining intensity was also increased in young HDFs co-cultured with A431 for 3 days when compared with young HDFs alone (Fig. S8C 9C).

In order to confirm the possible activation of NF-κB in HDFs during co-cultures, we performed western blot analyses of IκBα (NF-κB inhibitor alpha), which is one of the inhibitory proteins of NF-κB (Fig.10 and Fig. S12). In unstimulated cells, IκB interacts with NF-κB dimers in the cytoplasm, therefore preventing their nuclear translocation { ADDIN EN.CITE { ADDIN EN.CITE.DATA }}. However, upon NF-κB activation, IκB kinases (IKKs) trigger the phosphorylation and the proteasomal

degradation of I κ B (in particular I κ B α), which allows NF- κ B dimers to translocate to the nucleus and to activate target genes' transcription. As expected, we showed that I κ B α protein abundance in total protein extracts from TNF- α treated HDFs was significantly reduced, which provided a positive control for NF- κ B activation (Fig. 10A, C).

Interestingly, we also detected a significant decrease of the relative abundance of I κ B α in young and RS HDFs co-cultured with PM1, MET1 and MET4 cell lines, when compared with the control conditions alone after 6 days of co-culture (Fig. 10B, D). This suggests that I κ B α is degraded, allowing activation and nuclear translocation of NF- κ B. No significant impact of A431 cell line on I κ B α protein abundance in HDFs was detected (Fig. S12).

Altogether, these results are consistent with p65 nuclear translocation and the induction of SASP-related gene expressions in HDFs, which strongly suggests that NF- κ B is activated in HDFs during co-cultures with cSCC cell lines

4 Discussion

It is well known that senescent cells enhance growth, migration and invasion of cancer cells, notably through their SASP { ADDIN EN.CITE { ADDIN EN.CITE.DATA } } . However, little is known about their impact on cSCC carcinogenesis, which is linked with aging and cumulative sun exposure { ADDIN EN.CITE

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0065-2598 (Linking)</isbn><accession-num>25207363</accession-num><urls><related-urls><url>https://www.ncbi.nlm.nih.gov/pubmed/25207363</url></related-urls></urls></record></Cite></EndNote>}. We study here two relevant models of fibroblast senescence, which are replicative senescence and UVB-SIPS in dermal HDFs.

In senescence studies, attention should be paid to the use of relevant cellular senescence models, namely cell type and senescence inducer, which must be ideally selected according to the tissue context. Indeed, some senescence biomarkers, such as the SASP, might be highly modulated { ADDIN EN.CITE { ADDIN EN.CITE.DATA }}. As established that both chronological aging and UVR-induced photoaging, notably UVB, affect skin aging and cSCC development { ADDIN EN.CITE { ADDIN EN.CITE.DATA }}, two relevant models of fibroblast senescence were compared, namely RS and UVB-SIPS HDFs. We showed in this study that both models display a significant increase in some SASP-related gene expressions. Interestingly, all these genes have been previously mentioned in SASP studies, such as those performed including in senescent dermal BJ HDFs cells; { ADDIN EN.CITE { ADDIN EN.CITE.DATA }}

It is well documented that RS and SIPS share common senescence biomarkers { ADDIN EN.CITE <EndNote><Cite><Author>Toussaint</Author><Year>2002</Year><RecNum>30</RecNum><DisplayText>(Toussaint et al., 2002)</DisplayText><record><rec-number>30</rec-number><foreign-keys><key app="EN" db-id="e0vfs2zwqpxrpcewxt4p5ae4favdv5dxd5wp" timestamp="1499760995">30</key></foreign-keys><ref-type name="Journal Article">17</ref-type><contributors><authors><author>Toussaint, O.</author><author>Remacle, J.</author><author>Dierick, J. F.</author><author>Pascal, T.</author><author>Frippiat, C.</author><author>Royer, V.</author><author>Magalhacs, J. P.</author><author>Zdanov, S.</author><author>Chainiaux, F.</author></authors></contributors><auth-address>University of Namur, Unit of Cellular Biochemistry, Belgium. olivier.toussaint@fundp.ac.be</auth-

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SIPS HDFs display some common SASP-related gene expression changes, notably
 an increase in *IL1B* and *IL6* mRNA expression. In this regard, mass spectrometric
 analyses and cytokine multiplex assay ~~showed~~ have shown that IL-1 β is
 overexpressed in supernatant from intrinsically aged dermal HDFs { ADDIN EN.CITE
 { ADDIN EN.CITE.DATA }}. Moreover, a previous study has also ~~showed~~ described
 that *IL1B* mRNA level is increased in UVB-SIPS HDFs { ADDIN EN.CITE { ADDIN
 EN.CITE.DATA }}. For *IL6*, its mRNA level ~~was~~ is increased in RS dermal HDFs
 compared to young ones { ADDIN EN.CITE { ADDIN EN.CITE.DATA }} .
 Immunofluorescence staining have also showed that IL-6 expression in senescent
 stromal cells ~~was~~ is significantly increased in skin from old individuals compared to
 younger individuals { ADDIN EN.CITE { ADDIN EN.CITE.DATA }}. Finally, *IL6* mRNA
 and protein level ~~were~~ are ~~also~~ significantly increased in dermal HDFs after UVB

irradiation compared to non-irradiated HDFs { ADDIN EN.CITE

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Interestingly, we also demonstrated specific gene expression changes between our two models of senescence. ~~We highlighted~~ Indeed, a significant decrease in *CXCL1* and *CXCL2* gene expression in RS HDFs was highlighted. These decreases are also described in senescent HDFs from chronic wounds using Affymetrix microarray analysis and ELISA quantitation of conditioned medium { ADDIN EN.CITE { ADDIN EN.CITE.DATA }}. For UVB-SIPS HDFs, we showed an increase in *MMP1* and *MMP3* mRNA level. In this regard, it is known that dermis of photo-aged skin displayed higher level of *MMP1* protein level compared to sun-protected skin from the same individuals { ADDIN EN.CITE { ADDIN EN.CITE.DATA }}. Moreover, supernatant from UVB-irradiated dermal HDFs also displayed an increase in *MMP1* and *MMP3* protein level compared to non-irradiated HDFs { ADDIN EN.CITE

<EndNote><Cite><Author>Brenneisen</Author><Year>1999</Year><RecNum>36</RecNum><DisplayText>(Brenneisen et al., 1999)</DisplayText><record><rec-number>36</rec-number><foreign-keys><key app="EN" db-id="e0vfs2zwqpxrpcewxt4p5ae4favdv5dxd5wp"

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P.</author><author>Wlaschek, M.</author><author>Wenk, J.</author><author>Blaudschun, R.</author><author>Hinrichs, R.</author><author>Dissemond, J.</author><author>Krieg, T.</author><author>Scharffetter-Kochanek,

K. </author></authors></contributors><auth-address>Department of Dermatology, University of Cologne, Germany.</auth-address><titles><title>Ultraviolet-B induction of interstitial collagenase and stromelysin-1 occurs in human dermal fibroblasts via an autocrine interleukin-6-dependent loop</title><secondary-title>FEBS Lett</secondary-title></titles><periodical><full-title>FEBS Lett</full-title></periodical><pages>36-40</pages><volume>449</volume><number>1</number><keywords><keyword>Cells, Cultured</keyword><keyword>Child</keyword><keyword>Child, Preschool</keyword><keyword>Collagenases/*metabolism</keyword><keyword>Enzyme Induction</keyword><keyword>Humans</keyword><keyword>Interleukin-6/metabolism</keyword><keyword>Matrix Metalloproteinase 1</keyword><keyword>Matrix Metalloproteinase 3/*metabolism</keyword><keyword>Neutralization Tests</keyword><keyword>Ultraviolet Rays</keyword></keywords><dates><year>1999</year><pub-dates><date>Apr 16</date></pub-dates></dates><isbn>0014-5793 (Print)
0014-5793 (Linking)</isbn><accession-num>10225423</accession-num><urls><related-urls><url>https://www.ncbi.nlm.nih.gov/pubmed/10225423</url></related-urls></urls></record></Cite></EndNote>}. Finally, an increase in *CTGF*, *IGFBP3* and *TGFB1* mRNA level and both latent and active forms of TGFB1 was also described in UVB-SIPS HDFs { ADDIN EN.CITE { ADDIN EN.CITE.DATA } }.

The specific SASP-associated gene expression signature observed in our study between RS and UVB-SIPS HDFs is somewhat reminiscent of the “molecular scars” observed after between RS and subcytotoxic stresses as described previously { ADDIN EN.CITE { ADDIN EN.CITE.DATA } }. This reinforces the need to use complementary models of senescence according to the tissue context.

It is worth noting that the secretome of senescent HDFs has been previously involved in skin carcinogenesis. For instance, MMPs secreted by senescent dermal HDFs promoted post-senescence emergent keratinocytes migration, an early event in skin cancer development { ADDIN EN.CITE { ADDIN EN.CITE.DATA }}. Moreover, secreted frizzled-related protein 2 (sFRP2) in the secretome of HDFs from aged donors ~~promoted~~ induce melanoma cell invasion { ADDIN EN.CITE { ADDIN EN.CITE.DATA }}. In our study, we analyzed the impact of RS and UVB-SIPS HDFs conditioned media on growth, migration and invasion of cSCC cell lines but ~~we only observed~~ a limited impact was observed. These negative results might reflect the cSCC model, since we used skin-derived HDFs and cSCC cell lines. It is noteworthy that in a previous study, stimulation of keratinocytes with senescent HDFs conditioned media was achieved from the beginning of the culture until post-senescence emergence, which corresponds to about 45 days of stimulation { ADDIN EN.CITE { ADDIN EN.CITE.DATA }}. This long-term exposure, compared to short-term exposure, ~~promoted~~ promotes the pro-EMT effect of senescent fibroblasts' secretome on keratinocytes. This might partly explain ~~our limited observed~~ why we observed limited effects since cSCC cell lines were only exposed for 24 hours with conditioned media, even if short-term exposure remains the usual protocol { ADDIN EN.CITE { ADDIN EN.CITE.DATA }}.

~~We next analyzed~~ the impact of senescent HDFs ~~using~~ during indirect co-cultures was analyzed. ~~which~~ This allows bidirectional exchanges with cSCC cell lines. Again, a very limited impact of senescent HDFs on cSCC growth was detected.

Finally, we ~~then~~ analyzed the impact of cancer cell lines on HDFs, which is poorly investigated in senescence studies. Our results highlighted that cSCC cell lines in indirect co-cultures reinforce HDFs' senescent traits. Indeed, after co-culture with all cSCC cell lines, an increase in the proportion of SA- β gal positive cells ~~is~~ was

detected in young, but not in RS, HDFs when compared with the control condition. We next, studied SASP expression at the mRNA level was studied. Interestingly, our screening showed that co-cultures with PM1 and MET1 mostly induced a significant gene expression increase in young and RS HDFs, when compared with their respective controls (alone). These results were confirmed for *CCL2*, *IL1B* and *IL8* mRNA expressions on a larger sample size and with A431, another cSCC cell line. These over-expressions are particularly interesting given that these cytokines are widely known to be involved in carcinogenesis. Indeed, CCL2, also known as monocyte chemoattractant protein-1, has a key role in the infiltration of inflammatory cells and in tumor immunity { ADDIN EN.CITE

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specific protein-1) fibroblasts may maintain local inflammation, which promotes skin
carcinogenesis in DMBA/TPA- (7,12 Dimethylbenzanthracene/12-O-Tetradecanoyl
Phorbol 13-Acetate) induced skin cancer in mouse { ADDIN EN.CITE { ADDIN
EN.CITE.DATA }}. Regarding IL-1 β , which is a secreted form of IL-1, ~~which is~~
involved an involvement in inflammatory response { ADDIN EN.CITE
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Interestingly, these increases in pro-inflammatory genes expression after our co-cultures with cancer cells are consistent with a previous study, which highlighted

an education of dermal fibroblasts by carcinoma cells in K14/HPV16 mouse { ADDIN EN.CITE { ADDIN EN.CITE.DATA }}. These data showed that dermal fibroblasts displayed an induction of their inflammatory gene signature after incubation with conditioned media from a skin carcinoma cell line *ex vivo* and *in vivo*.

Moreover, the over-expressions of SASP-related genes observed in our study were more pronounced in co-culture with MET1, the highly invasive cSCC cell line. In this regard, CCL2, IL-1 β and IL-8 were also up-regulated at the mRNA and protein levels in dermal HDFs co-cultured or exposed to conditioned media from several invasive melanoma cell lines when compared to a non-invasive one { ADDIN EN.CITE { ADDIN EN.CITE.DATA }}. In this respect, this entails an increase in the invasive potential of melanoma cell lines upon co-culture with HDFs compared to stimulation with fibroblast-conditioned media. These bidirectional interactions are also described between cancer-associated fibroblasts and tumor cells, which involved chemokines { ADDIN EN.CITE

<EndNote><Cite><Author>Mishra</Author><Year>2011</Year><RecNum>47</RecNum><DisplayText>(Mishra et al., 2011)</DisplayText><record><rec-number>47</rec-number><foreign-keys><key app="EN" db-id="e0vfs2zwqpxrpcewxt4p5ae4favdv5dxd5wp" timestamp="1500294697">47</key></foreign-keys><ref-type name="Journal Article">17</ref-type><contributors><authors><author>Mishra, P.</author><author>Banerjee, D.</author><author>Ben-Baruch, A.</author></authors></contributors><auth-address>Department of Medicine and Pharmacology, The Cancer Institute of New Jersey, Robert Wood Johnson Medical School University of Medicine and Dentistry of New Jersey, New Brunswick, New Jersey, USA.</auth-address><titles><title>Chemokines at the crossroads of tumor-fibroblast interactions that promote malignancy</title><secondary-title>J Leukoc

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 observed in our study.

~~We next studied~~ The mechanisms underlying these SASP factors over-
 expressions during co-culture were then investigated. ~~We detected a significant~~
~~increase in nuclear p65 staining in young and RS HDFs co-cultured with PM1 and~~
~~MET4.~~ This Our results strongly suggests that NF-κB, a master regulator of the
 mRNA expression of most SASP factors { ADDIN EN.CITE
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cultures. Indeed, we detected a significant increase in nuclear p65 staining in young
and RS HDFs co-cultured with PM1 and MET4. Interestingly, an increase in nuclear
p65 staining in stromal fibroblasts ~~was~~ is also observed in skin section from
K14/HPV16 mouse but not in control mice skin { ADDIN EN.CITE { ADDIN
EN.CITE.DATA }}. ~~In our study, HDFs co-cultured with MET1 were not associated~~

~~with NF- κ B activation.~~ A significant decrease of I κ B α , which is reminiscent of NF- κ B activation { ADDIN EN.CITE { ADDIN EN.CITE.DATA } }, was also observed in protein extracts from young and RS HDFs co-cultured with PM1, MET1 and MET4. ~~Thus~~ However, others mechanisms might also be involved in SASP expression, such as the increased activity of C/EBP β (CCAAT/enhancer binding protein beta), the other major transcription factor involved in senescence secretome in ~~SASP~~ expression { ADDIN EN.CITE { ADDIN EN.CITE.DATA } }. The possible signaling between cSCC cell lines and HDFs is presented in Fig. 11

In summary, our results highlighted cellular interactions within cSCC microenvironment and also the need to use co-culture experiments in addition to conditioned media approaches. These data strongly suggest that signaling from cSCC cell lines induce senescence and SASP expression in young HDFs and reinforce SASP expression in RS HDFs. The question that remains open is if these results might explain the accumulation of senescent cells at site of malignant transformation { ADDIN EN.CITE { ADDIN EN.CITE.DATA } }. Overall, understanding the interplay between tumor cells and their microenvironment may have important outcomes in cancer research and therapeutic strategies.

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Conflicts of interest

The authors declare no conflict of interest.

Author Contributions

M.T. designed, performed and analyzed the experiments and wrote the manuscript; E.D., A.F. and M.VS. performed experiments; C.M.P. provided the PM1, MET1 and MET4 cell lines and revised the manuscript; F.D-C supervised the work and revised the manuscript.

Reference list

{ ADDIN EN.REFLIST }

Captions of figures

Supplementary Fig. S2-Fig. 1. *Impact of senescent dermal HDFs-conditioned media on cutaneous squamous cell carcinoma (cSCC) cell lines growth.*

Conditioned media were collected after 48 hours of incubation with media alone (BME) or from young (Y), replicative senescent (RS), control of stress-induced premature senescent (CTL-SIPS) or SIPS human diploid fibroblasts (HDFs). (A) PM1, (B) MET1 and (C) MET4 cell growths were measured after 24 hours of incubation with conditioned media using MTS assay. They show the mean percentage of cell growth when compared with BME $\pm$ SEM from three independent experiments with technical triplicates (** $p < 0.01$, *** $p < 0.001$).

Supplementary Fig. S3-Fig. 2. *Impact of senescent dermal HDFs-conditioned media on cutaneous squamous cell carcinoma (cSCC) cell lines migration.*

Conditioned media were collected after 48 hours of incubation with media alone (BME) or from young (Y), replicative senescent (RS), control of stress-induced premature senescent (CTL-SIPS) or SIPS human diploid fibroblasts (HDFs). (A) PM1, (B) MET1 and (C) MET4 migrating cells were counted after 24 hours of incubation with conditioned media as attractant using Boyden Chambers. It is represented as the mean percentage when compared with BME $\pm$ SEM from three independent experiments with technical triplicates (* $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$).

Supplementary Fig. S4-Fig. 3. *Impact of senescent dermal HDFs-conditioned media on cutaneous squamous cell carcinoma (cSCC) cell lines invasion.*

Conditioned media were collected after 48 hours of incubation with media alone (BME) or from young (Y), replicative senescent (RS), control of stress-induced premature senescent (CTL-SIPS) or SIPS human diploid fibroblasts (HDFs). (A)

MET1 and (B) MET4 invading cells were counted after 24 hours of incubation with conditioned media as attractant using matrigel coated-Boyden Chambers. It is represented as the mean percentage when compared with BME $\pm$ SEM from three independent experiments with technical triplicates (** $p < 0.01$, *** $p < 0.001$).

Fig. 1 4. *Experimental design and time frame of the indirect co-cultures.*

Young dermal human diploid fibroblasts (HDFs) were cultivated during several months until replicative senescence. UVB-stress-induced premature senescence (UVB-SIPS) HDFs were obtained according to the protocol described in (Debacq-Chainiaux et al., 2005). Briefly, young HDFs were exposed to UVB (250 mJ/cm²) twice a day during five days and then collected after 72h of recovery. For indirect co-culture experiments, HDFs were seeded into wells at day -1. Cutaneous squamous cell carcinoma (cSCC) cell lines were seeded into transwells at day 0. Co-cultures analyses were then performed at day 1, 3 and 6. Culture media were changed on day 3.

Fig. 2 5. *Impact of senescent dermal HDFs-co-cultures on cutaneous squamous cell carcinoma (cSCC) cell lines growth.*

(A) PM1, (B) MET1 and (C) MET4 relative growths were measured after 1, 3 and 6 days of co-culture using MTS assay. Co-cultures were performed with young (+ Y), replicative senescent (+ RS), control of stress-induced premature senescent (+ CTL) or SIPS human diploid fibroblasts (HDFs) using transwell. Results show the relative growth ratio (when compared with Y or CTL at day 1) $\pm$ SEM from four independent experiments for RS HDFs-co-cultures and three independent experiments for SIPS HDFs-co-cultures (* $p < 0.05$, ** $p < 0.01$).

Supplementary Fig. S7 Fig. 6. *Impact of cutaneous squamous cell carcinoma (cSCC) cell lines-co-cultures on percentage of senescence-associated- β galactosidase (SA- β gal) positive dermal HDFs.*

Young (Y) or replicative senescent (RS) human diploid fibroblasts (HDFs) were maintained in co-culture alone or with PM1, MET1 or MET4 using transwell. At day 1 and 6 of co-culture, HDFs were reseeded and stained for SA- β gal activity the next day. Results are represented as the mean percentage of SA- β gal positive HDFs $\pm$ SEM (400 cells were counted per condition) from one experiment with technical triplicates (# comparison between alone and +PM1/+MET1/+MET4, *,#p<0.05, **p<0.01,###,***p<0.001).

Fig. 3 7. *Impact of cutaneous squamous cell carcinoma (cSCC) cell lines-co-cultures on senescence-associated secretory phenotype (SASP) mRNA abundances in young and replicative senescent dermal HDFs.*

(A) *CCL2*, (B) *IL1B* and (C) *IL8* relative mRNA abundances. Young (Y) or replicative senescent (RS) human diploid fibroblasts (HDFs) were maintained in co-culture alone or with PM1, MET1 or MET4 using transwell. At day 1 and 6 of co-culture, relative mRNA abundances were measured by RT-qPCR. Fold changes are normalized to the mRNA abundance of glyceraldehyde-3-phosphate dehydrogenase (*GAPDH*), and are expressed relative to Y alone at day 1. Results are represented as the mean log2 fold change $\pm$ SEM from four independent experiments (# comparison between alone and +PM1/+MET1/+MET4, *,#p<0.05, ##p<0.01, **,\$p<0.05, ***,\$p<0.05,###,***p<0.001).

Fig. 4 Fig. 8. *Impact of A431-co-culture on SASP mRNA abundances in young and replicative senescent dermal HDFs. (A) CCL2, (B) IL1B and (C) IL8 relative mRNA abundances.*

Young (Y) or replicative senescent (RS) human diploid fibroblasts (HDFs) were maintained in co-culture alone or with A431 using transwell. At day 1 and 3 of co-culture, relative mRNA abundances were measured by RT-qPCR. Fold changes are normalized to the mRNA abundance of glyceraldehyde-3-phosphate dehydrogenase (*GAPDH*), and are expressed relative to Y alone at day 1. Results are represented as the mean log2 fold change $\pm$ SEM from two independent experiments in technical duplicates (*, #p<0.05, **p<0.01, ***p<0.001).

Supplementary Fig. S8 Fig. 9. *Impact of cutaneous squamous cell carcinoma (cSCC) cell lines-co-cultures on nuclear p65 intensity in young and replicative senescent dermal HDFs.*

(A) Representative immunofluorescence staining images and quantification of nuclear p65 intensity in negative and positive controls (fibroblasts incubated without or with tumor necrosis factor- α) are shown. Montage images (of 3 x 3 containing 9 adjacent image fields) were acquired using a 20X objective. (B) Immunofluorescence staining quantification in PM1-, MET1- and MET4-co-cultures. (C) Immunofluorescence staining quantification in A431-co-cultures. Young (Y) and replicative senescent (RS) human diploid fibroblasts (HDFs) were maintained in co-culture alone or with PM1, MET1, MET4 (for 6 days) or A431 (for 3 days) using transwell. p65 fluorescence intensity was measured in the nucleus using BD Pathway 855. Results are represented as the mean fluorescence intensity per cell $\pm$ SEM (an average of 250 cells was counted per condition) from one experiment (# comparison between alone and +PM1/+MET1/+MET4/+A431, \$\$p<0.01, ***,# #,\$\$\$p<0.001).

Fig. 10. *Impact of cutaneous squamous cell carcinoma (cSCC) cell lines-co-cultures on I κ B α protein abundance in young and replicative senescent dermal HDFs.*

Representative western blot analysis of I κ B α (nuclear factor-kappa B inhibitor alpha) in (A) negative and positive controls (fibroblasts incubated without or with tumor necrosis factor-alpha) and (B) young (Y) or replicative senescent (RS) human diploid fibroblasts (HDFs) maintained in co-culture alone or with PM1, MET1, MET4 for 6 days using transwell. β -actin was used as loading control. Results originate from the same immunoblot at the indicated molecular weight (MW). (C-D) Quantification of the relative abundance of I κ B α . Signal intensities were quantified, normalized to the abundance of β -actin, and are expressed relative to CTL - (C) or Y alone (D). Results are represented as the mean fold change $\pm$ SEM from technical duplicates (C) or triplicates (D) (# comparison between alone and +PM1/+MET1/+MET4, * p <0.05, # p <0.01, ### p <0.001).

Fig. 11. *Possible signaling between cutaneous squamous cell carcinoma (cSCC) cell lines and dermal HDFs.*

Co-cultures between cSCC cell lines and dermal human diploid fibroblasts (HDFs) allow bidirectional exchanges. Extracellular signaling from cSCC cell lines induces senescence and senescence-associated secretory phenotype (SASP) expression in young HDFs and reinforces SASP in replicative senescent HDFs. This might involve an activation of nuclear factor-kappa B (NF- κ B) and/or other transcription factors such as C/EBP β (CCAAT/enhancer binding protein beta) in HDFs. It results in cSCC cell lines growth, and may impact migration and invasion. Overall these exchanges are modulated by the SCC cell lines' tumorigenesis stage.

Tables

Table 1. Screening of the senescence-associated secretory phenotype (SASP)-related mRNA abundances in senescent dermal HDFs.

Gene	Name	Fold change	p-value	
(A)		RS		
<i>IL1B</i>	Interleukin 1 beta	9.08	0.011	*
<i>ICAM1</i>	Intercellular adhesion molecule 1	2.65	0.012	*
<i>CTGF</i>	Connective tissue growth factor	2.56	0.001	**
<i>IL6</i>	Interleukin 6	2.52	0.002	**
<i>CXCL2</i>	C-X-C motif chemokine ligand 2	0.46	0.027	*
<i>IGFBP2</i>	Insulin like growth factor binding protein 2	0.40	0.052	*
<i>CXCL1</i>	C-X-C motif chemokine ligand 1	0.37	0.001	**
<i>HGF</i>	Hepatocyte growth factor	0.31	0.010	*
<i>MMP3</i>	Matrix metalloproteinase 3	0.27	0.005	**
(B)		SIPS		
<i>MMP3</i>	Matrix metalloproteinase 3	8.46	0.003	**
<i>CXCL1</i>	C-X-C motif chemokine ligand 1	4.82	0.000	***
<i>IL1B</i>	Interleukin 1 beta	3.05	0.030	*
<i>CTGF</i>	Connective tissue growth factor	2.95	0.002	**
<i>IL8</i>	Interleukin 8	2.71	0.048	*
<i>CCL2</i>	C-C motif chemokine ligand 2	2.58	0.013	*
<i>FGF2</i>	Fibroblast growth factor 2	2.57	0.002	**
<i>MMP1</i>	Matrix metalloproteinase 1	2.41	0.022	*
<i>IL6</i>	Interleukin 6	2.37	0.047	*
<i>IGFBP6</i>	Insulin like growth factor binding protein 6	2.05	0.024	*
<i>IGFBP3</i>	Insulin like growth factor binding protein 3	1.81	0.009	**
<i>CXCL2</i>	C-X-C motif chemokine ligand 2	1.69	0.005	**
<i>TIMP2</i>	TIMP metalloproteinase inhibitor 2	1.61	0.001	**
<i>TGFB1</i>	Transforming growth factor beta 1	1.61	0.049	*

(A) Relative mRNA abundances in replicative senescent (RS) HDFs. (B) Relative mRNA abundances in UVB stress-induced premature senescent (SIPS) HDFs. Relative mRNA abundances were measured using TaqMan Low Density Arrays (TLDA) from five independent experiments for RS and from four independent experiments for SIPS. Fold changes are normalized to the mRNA abundance of glyceraldehyde-3-phosphate dehydrogenase (*GAPDH*), and are expressed relative to young or CTL-SIPS HDFs. The significant changes are represented (* $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$).

Table 2. Screening of senescence-associated secretory phenotype (SASP)-related mRNA abundances in young and replicative senescent dermal HDFs after 6 days of co-culture with cutaneous squamous cell carcinoma (cSCC) cell lines.

Gene	Name	Fold change (relative to Y alone at day 1)						Impact of senescence		Impact of co-culture		Post hoc test for co-culture
		Y alone	Y + PM1	Y + MET1	RS alone	RS + PM1	RS + MET1					
<i>CCL2</i>	C-C motif chemokine ligand 2	4.7	22.1	42.8	15.5	63.5	82.7	↑	*	↑	*	+ PM1 (#), + MET1 (#)
<i>CXCL2</i>	C-X-C motif chemokine ligand 2	2.6	32.1	38.6	4.2	60.5	69.7	↑	**	↑	***	+ PM1 (###), + MET1 (###)
<i>IL1B</i>	Interleukin 1 beta	0.4	4.7	5.7	3.4	19.7	25.5	↑	*	↑	*	+ PM1 (#), + MET1 (#)
<i>MMP1</i>	Matrix metalloproteinase 1	0.0	0.1	0.1	0.2	0.4	0.5	↑	**	↑	*	+ PM1 (#), + MET1 (#)
<i>TIMP2</i>	TIMP metalloproteinase inhibitor 2	1.5	1.3	1.3	1.8	1.6	1.7	↑	**	↓	*	+ PM1 (#)
<i>CCL20</i>	C-C motif chemokine ligand 20	7.5	67.7	122.8	11.9	347.2	349.5	NS		↑	*	+ PM1 (#), + MET1 (#)
<i>CXCL1</i>	C-X-C motif chemokine ligand 1	1.1	45.2	91.6	0.5	50.8	87.7	NS		↑	*	+ PM1 (#), + MET1 (#)
<i>ICAM1</i>	Intercellular adhesion molecule 1	7.6	17.3	22.5	9.7	17.1	19.4	NS		↑	*	+ MET1 (#)
<i>IL8</i>	Interleukin 8	0.9	51.6	92.9	0.8	141.3	175.0	NS		↑	*	+ PM1 (#), + MET1 (#)
<i>FGF2</i>	Fibroblast growth factor 2	0.5	0.7	0.6	1.0	1.0	1.1	↑	*	NS		NS
<i>IGFBP3</i>	Insulin like growth factor binding protein 3	0.9	0.9	0.8	2.5	1.8	2.6	↑	*	NS		NS
<i>IGFBP5</i>	Insulin like growth factor binding protein 5	0.2	0.2	0.2	0.4	0.3	0.3	↑	*	NS		NS
<i>MMP3</i>	Matrix metalloproteinase 3	0.0	0.1	0.1	0.4	1.6	1.3	↑	*	NS		NS
<i>MMP10</i>	Matrix metalloproteinase 10	0.1	0.1	0.1	0.4	0.2	0.3	↑	*	NS		NS
<i>TNFRSF11B</i>	TNF receptor superfamily member 11B	1.1	0.9	1.1	3.7	2.6	2.8	↑	**	NS		NS
<i>IGFBP2</i>	Insulin like growth factor binding protein 2	5.5	5.8	4.7	2.8	1.6	1.8	↓	*	NS		NS
<i>PTGS1</i>	Prostaglandin-endoperoxide synthase 1	0.4	0.7	0.4	0.1	0.1	0.1	↓	**	NS		NS
<i>VCAM1</i>	Vascular cell adhesion molecule 1	19.7	33.0	32.6	5.7	13.0	13.6	↓	*	NS		NS

Young (Y) or replicative senescent (RS) HDFs were maintained in co-culture alone or with PM1 or MET1 using transwell. Relative mRNA abundances were measured using TaqMan Low Density Arrays (TLDA) from one experiment. Fold changes are normalized to the mRNA abundance of glyceraldehyde-3-phosphate dehydrogenase (*GAPDH*) and *RNA18S*, and are expressed relative to Y alone at day 1 of co-culture. The significant impact of senescence and / or co-culture is represented in three categories: impact of

senescence and co-culture; impact of co-culture and impact of senescence (↑ upregulation, ↓ repression, NS non significant). If co-culture impact was significant, Tukey was used as *post hoc* test (# comparison between alone and + PM1 or + MET1). *,#p<0.05, **p<0.01, ***,# #p<0.001

FIG1 (anciennement FIGS2)

FIG2 (anciennement FIGS3)

FIG3 (anciennement FIGS4)

FIG4 (anciennement FIG1)

FIG5 (anciennement FIG2)

FIG6 (anciennement FIGS7)

FIG7 (anciennement FIG3)

FIG8 (anciennement FIG4)

FIG9 (anciennement FIGS8 avec ajout des photos)

A.

B.

C.

FIG10 (new figure)

FIG11 (new schematic representation)

PM1

MET1

MET4

A431

MDA-MB-231

FIGS2 (anciennement FIGS1)

Y

RS

CTL-SIPS

SIPS

FIGS4 (new FIG)

A. (PM1)

B. (MET1)

C. (MET4)

A. (MET1)

B. (MET4)

FIGS6 (anciennement FIGS5) paysage

FIGS7 (anciennement FIGS6) paysage

SIPS HDFs-co-cultures

Day 6

$$RS +$$

Day 6

+ CTL-SIPS

+ SIPS

Day 1

Day 6

$$\begin{array}{c} \succ \\ + \end{array}$$
$$S$$

Day 1

Day 6

+ CTL-SIPS

SIPS +

Day 1

Day 6

$$+ \gamma$$
$$S$$

Day 1

Day 6

+ CTL-SIPS

SIPS
+

FIGS9 (NEW FIG) paysage

FIGS10 (new FIG)

A. (Day 1)

B. (Day 6)

FIGS11 (new FIG)

A. (Day 1)

B. (Day 3)

A.

B.

Human dermal fibroblasts

INTERPLAY

Cutaneous SCC cell lines
at different stages of
tumorigenesis

FIGS6 (anciennement FIGS5 avec ajout des photos)

A.

B.

C.

FIGS7 (anciennement FIGS6 avec ajout des photos)

A.

B.

FIGS9 (new FIG)

A.

B.