

**Chronic resveratrol treatment inhibits MRC5 fibroblast
SASP-related pro-tumoral effects on melanoma cells**

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3 **Chronic resveratrol treatment inhibits MRC5 fibroblast SASP-related pro-**
4 **tumoral effects on melanoma cells**
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55 Running headline: Resveratrol modulates pro-tumoral SASP
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Abstract

Cellular senescence is related to organismal aging and is observed after DNA damaging cancer therapies, that induce tumor-suppressive modifications, but it is characterized by a strong increase in secreted factors, termed the “senescence-associated secretory phenotype” (SASP). Particularly, SASP from stroma senescent fibroblasts creates a cancer-favoring microenvironment, providing targets for anti-cancer interventions. In the present paper, chronic treatment (5 weeks) with 5 μ M resveratrol has been used to modulate senescence-related pro-tumoral features of MRC5 fibroblasts, reducing SASP-related interleukins IL1 α , IL1 β , IL6 and IL8; transforming-growth-factor- β (TGF β); matrix metallo-proteinases MMP3 and MMP2; urokinase plasminogen activator (uPA); receptor proteins uPAR, IL6R, insulin growth factor receptor-1 (IGF-1R), TGF β -R2, and CXCR4. The cellular nuclear-factor-kB (NF-kB) protein level was also reduced, confirming its role in the induction of SASP. Resveratrol pre-treated MRC5 fibroblasts were resistant to activation by TGF β . Resveratrol treatment of senescent MRC5 induced the production of conditioned media (CM) which counteracted the pro-tumoral effect of senescent CM on A375 and A375-M6 melanoma cell proliferation and invasiveness, and reduced the expression of epithelial-to-mesenchymal transition markers related to malignant features. This experimental approach proposes a treatment that targets the senescent stromal cell phenotype to induce an anti-tumor hosting microenvironment, which is suitable for both preventive and therapeutic purposes.

Keywords: Resveratrol, Cellular senescence, Cancer

INTRODUCTION

Cell senescence is a complex process that modifies the protein expression profile of cells and leads to replicative arrest and changes in metabolism, adhesion efficiency and secretion phenotype (1). Cell senescence is accompanied by a striking increase in the secreted levels of approximately 40 factors involved in intercellular signaling, termed the “senescence-associated secretory phenotype” (SASP, 2). Cellular senescence response is considered an efficacious tumor suppressive mechanism, preventing at-risk cells, such as DNA damaged (3) cells, from undergoing malignant transformation. Some SASP components may reinforce these positive senescence features; however, when chronically present, SASP may be deleterious, stimulating age-associated tissue degeneration or creating a tumor-favoring tissue microenvironment, which promotes malignant phenotypes *in vitro* and *in vivo* (see reviews by Campisi and co-workers, 4,5). SASP includes factors that may activate receptors and signaling pathways of neighbor cells and can be divided into the following three categories: i) soluble signaling factors (interleukins, chemokines, and growth factors), ii) secreted proteases and iii) insoluble components. The SASP mediators relevant for pro-tumoral activity are as follows: i) inflammatory cytokines, affecting both endothelial and epithelial cells (6) by activating the nuclear factor kappa-light-chain-enhancer of activated B cells (NF- κ B) pathway (7) and inducing epithelial-mesenchymal transition (EMT) (4); ii) members of CXCL and CCL families like IL-8, growth related oncogene-alpha ($\text{GRO}\alpha$) and β , stromal derived factor1 (SDF1) and its receptor CXCR4 (8); iii) matrix metallo-proteinases (MMPs) that alter the extracellular matrix (6) and have been showed to support mitogenic and chemotactic signal access to breast cancer cells (4); iv) urokinase-plasminogen-activator/urokinase-plasminogen-activator receptor (uPA/uPAR) system (9,10); v) almost all insulin-like growth factor (IGF)-binding proteins and their regulators (6). In addition, SASP-expressing senescent cells can exacerbate tumor immunoediting and eventually allow for the growth of immune-resistant cancer cells (11).

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3 Fibroblasts are the main components of tissue stroma and their products are essential for
4 remodeling and repair processes, but when activated or once senescent, they can contribute to
5 progression and invasion of cancer cells, which is partially due to certain pro-inflammatory SASP
6 factors, such as IL-6, IL-8 and GRO α (12). Additionally, cancer-associated fibroblasts show a
7 secretory pattern resembling SASP (10). This is the rationale for targeting senescent fibroblasts in
8 tumor stroma to increase the effectiveness of cancer therapy (13).
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16 A wide range of activities has been conferred to polyphenols, which are capable of modulating
17 factors that are altered during aging and are relevant for age-related diseases. Polyphenols are
18 widely distributed among dietary fruits and vegetables; their consumption has been related to a
19 decreased risk of cardiovascular, metabolic and neurodegenerative diseases, as well as cancer.
20 Certain compounds, such as curcumin, epigallocatechin and resveratrol, have been proposed as
21 potential anticancer therapeutics, alone or in combination with classical chemotherapy, by
22 interacting with multiple signaling pathways, including the NF-kB pathway. These compounds also
23 modulate cell cycle and apoptosis (targeting p53 and the anti-apoptotic bcl-2 family members) and
24 angiogenesis (targeting VEGF and FGF) (all reviewed by Fantini et al, 14).
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36 Among the polyphenolic compounds, resveratrol (*trans*-3,4,5-trihydroxystilbene) has been
37 widely studied for its anti-senescence and anti-tumor properties (15), which are related to sirtuin1
38 activation and anti-oxidant features. Recently, resveratrol was reported to inhibit, through the above
39 cited pathways, EMT in prostate, ovarian and colorectal cancers as well as to synergize with
40 tamoxifen in antiestrogen-resistant breast cancer cells. (16)
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3 Previous studies conducted in our lab (17,18) evaluated the effects of a long-term treatment (5
4 weeks) with low resveratrol concentrations (1 and 5 μ M) in human MRC5 fibroblasts, a well-
5 known *in vitro* senescence model. These concentrations are compatible with those attainable *in vivo*
6 and are fairly maintained in culture (17). The treatment reverted some senescence-related
7 morphologic and functional alterations in pre-senescent MRC5 cells (17) as well as inhibited gene
8 expression and secretion of some SASP-related inflammatory molecules (18). In the present study,
9 we used the forementioned chronic treatment with 5 μ M resveratrol (reported as R5) of pre-
10 senescent MRC5 fibroblast cultures progressing towards replicative senescence, to modulate the
11 expression and release of SASP factors.
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22 It is well-known that transforming growth factor- β 1 (TGF β 1) mediates fibroblast activation to
23 myofibroblasts (19), playing a central role in the development of a pro-tumoral microenvironment.
24 Additionally, the effect of R5 treatment on MRC5 fibroblast activation by TGF β 1 was evaluated.
25 Furthermore, to mimic an anti-tumor hosting microenvironment *in vitro*, conditioned media (CM)
26 from pre-treated fibroblast cultures were used to assay their ability to modulate tumor-related
27 phenotypes of A375 and A375-M6 melanoma cell lines.
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38 METHODS

39 Cell cultures and treatments

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43 The MRC5 cell line is a normal human fibroblast cell line derived from foetal lung, and it was
44 purchased from National Institute of Aging Cell Repository (Coriell Institute, USA). The A375
45 human melanoma cell line (MITF wild type, BRAF V600E, NRAS wild type) was obtained from
46 American Type Culture Collection (Manassas, VA), and A375-M6 melanoma cells were isolated
47 from lung metastasis of SCID bg/bg mice that were i.v. injected with A375 and gently provided by
48 Prof. Calorini (University of Florence). MRC5 fibroblasts and melanoma cells were cultured in
49 high-glucose (4,500 mg/L) Dulbecco's Modified Eagle's Medium (DMEM, Euroclone) that was
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3 supplemented with 10% foetal bovine serum (FBS, Sigma–Aldrich), 2 mM L-glutamine, 100
4 units/mL penicillin and 100 µg/mL streptomycin (Sigma–Aldrich) at 37°C in a 5% CO₂ humidified
5 atmosphere. Cells were propagated at confluence by trypsinization.
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9 The experiments on MRC5 fibroblasts were conducted on pre-senescent cultures, defined as
10 having undergone more than 45 PDL. After chronic treatment for 5 weeks with or without 5 µM
11 final resveratrol concentration (Sigma–Aldrich) and propagation in culture, with medium changes
12 every two days, **pre-senescent cells** became senescent, as described previously (17,18). At the end
13 of the treatment, control and resveratrol-treated senescent MRC5 cultures were named and reported
14 as sen and senR5, respectively. Low PDL (< 30) MRC5 fibroblasts were also propagated in culture
15 and reported as young.
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18 In some experiments, at the end of the 5 week treatment, **sen and senR5** cell cultures were
19 subsequently incubated for 24h in fresh DMEM added with transforming-growth-factor-beta 1
20 (TGFβ1, PeproTech) at a concentration of 10ng/mL.
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23 **Preparation of MRC5 conditioned media**

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25 **At the end of treatments, cell cultures were washed with fresh PBS, next** conditioned media
26 **(CM)** were collected after 24 h of incubation with DMEM plus 2% FBS, and centrifugation at
27 1,500 rpm for 5 minutes. The medium volume/cell number proportion was 1 mL/ 1x10⁵ MRC5
28 fibroblasts.
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31 **A375 and A375-M6 treatment with MRC5 CMs**

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33 **Melanoma** cells were seeded at density of 2.0 x 10⁴ cells/cm²; once grown to approximately
34 80% confluency cells were incubated for 48 h with unconditioned DMEM plus 2% FBS, reported as
35 DMEM, or with CM from sen and senR5 MRC5. Then, cells were recovered by trypsinization for
36 Real Time PCR (RT PCR), western blot analyses, or invasion assays. Media for zymographies were
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3 collected from A375 and A375-M6 cultures that were subsequently incubated for 24 h with fresh
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5 unconditioned DMEM. The medium volume/cell number proportion was 1mL/1.5x10⁶ cells.
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8 9 **A375 and A375-M6 growth determination**

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11 **Melanoma** cells were seeded at a density of 2 x 10⁴ cells/cm² and incubated with either DMEM
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13 or CM from **either** sen or senR5 fibroblasts. At 24, 48 **and 72 h**, cells were recovered by
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15 trypsinization and counted in a Bürker chamber. Cell viability was assessed by the Trypan Blue
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17 exclusion test.
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20 21 **Real Time PCR**

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23 Total RNA extraction was conducted using TRIzol reagent (Invitrogen). RNA was reverse
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25 transcribed with an iScript cDNA Synthesis Kit using random primers. mRNA expression of
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27 selected genes was assayed by Real Time PCR using the primers (IDT, TemaRicerca) listed in table
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29 1 (supplementary materials 1). Quantitative RT PCR was performed with an Applied Biosystem
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31 7500 Fast RT PCR System (Applied Biosystem) using a SYBR Green-based detection with the
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33 default PCR settings as follows: 40 cycles of 95°C for 15s and of 60°C for 60s. The “Delta-delta
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35 method” was used for comparing relative gene expression results with GAPDH as the housekeeping
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37 gene. Data were normalised to results obtained in young, untreated MRC5 (sen), or in **melanoma**
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39 cells incubated with fresh unconditioned DMEM with 2% FBS (in both **all** cases, assumed as value
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41 100%) and expressed as the means ± standard deviation (SD) of 3 experiments.
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49 50 **Western Blot analyses**

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52 Cell aliquots of MRC5, **A375 and** A375-M6 cells were collected at the end of the respective
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54 treatments and lysed in RIPA buffer (25mM Tris-HCl pH 7.6, 150mM NaCl, 1% NP-40, 1%
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56 sodium deoxycholate, 0.1% sodium dodecyl sulfate, SDS) with 1% protease inhibitor Cocktail
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58 (Sigma-Aldrich Chemicals Co.) and disrupted by sonication (Microson XL-2000, Misonix). Lysates
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3 were clarified by centrifugation and supernatant collected and stored at -20°C. Protein content was
4
5 estimated by the Bio-Rad DC protein assay kit. Protein aliquots (30 to 40 µg), were separated using
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7 12% SDS-polyacrylamide gel electrophoresis (SDS-PAGE, NuPAGE, Novex; Invitrogen),
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9 transferred to nitrocellulose membranes (Millipore), blocked in 5% skim milk and incubated
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11 overnight with the following specific primary antibodies: mouse anti-tubulin (clone DM 1A, T 9026
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13 Sigma-Aldrich Chemicals Co.), mouse anti- α -smooth muscle actin (α -SMA, clone 1A4, A2547,
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15 Sigma-Aldrich Chemicals Co.), rabbit anti-NF- κ B (GTX102090, GeneTex, Irvine, California),
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17 mouse anti-uPAR (10G7, sc-13522, Santa Cruz, Santa Cruz, California) that recognizes the full-
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19 length uPAR, rabbit anti-transforming growth factor β -receptor 2 (TGF β -R2, 3713, Cell Signaling),
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21 rabbit anti-phospho-p38 mitogen activated protein kinase (P-p38, 92156, Cell Signaling), rabbit
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23 anti-p38 (44-6846, Biosource), rabbit anti-phospho-extracellular signal-regulated kinase (P-ERK,
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25 p42/p44, 9101, Cell Signaling), rabbit anti-ERK-2 (sc-154, Santa Cruz), mouse anti-N-cadherin (N-
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27 cad, clone 6G11, Dako), and rabbit anti-CXCR4 (AB 1846, Millipore), followed by the suitable
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29 HRP-conjugated secondary antibodies (Sigma-Aldrich Chemicals Co.). All resulting
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31 immunocomplexes were visualized with an enhanced chemiluminescence ECL detection system
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33 (GE Healthcare) and quantified by ImageJ software (NIH).
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42 **Invasion assay**

43 Analyses were performed in Boyden chambers, with wells separated by 8 µm-pore size
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45 polycarbonate filters coated with Matrigel (50µg/filter). In chemoinvasion experiments, 2×10^4 A375
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47 and 10^4 A375-M6 melanoma cells were suspended in 200 µL of DMEM plus 2% FBS and placed in
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49 upper well towards 200 µL of either MRC5 conditioned media or unconditioned DMEM plus 2%
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51 FBS (used as reference) which was placed in the lower well. In spontaneous invasion experiments,
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53 subconfluent A375 and A375-M6 cultures were incubated for 48 h with DMEM or MRC5
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55 conditioned media. Next, 2×10^4 cells from A375 cultures and 10^4 cells from A375-M6 cultures of
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57 each incubation group were suspended in 200 µL of fresh DMEM plus 2% FBS, respectively, and
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3 placed in the upper wells of Boyden chambers. Fresh DMEM plus 2% FBS was also placed in the
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5 lower well.

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7 In both experiment types, invasion was performed for 6 hours at 37°C in 5% CO₂, then filters
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9 were recovered and fixed in methanol. Non-invading cells on the upper surface of the filter were
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11 removed with a cotton swab while invasive cells that were adherent on the lower filter surface were
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13 stained and counted using a light microscope.

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15 For the chemoinvasion assay, the results were expressed as the percentage of cells that migrated
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17 towards MRC5 CMs compared to those that migrated towards DMEM. For spontaneous invasion
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19 activity determination, results were reported as the percentage of melanoma cells that migrated after
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21 pre-incubation with MRC5 CMs compared to those that migrated after pre-incubation with DMEM.
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27 **Zymography**

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29 Aliquots (30 µL) of CM obtained as described above from MRC5, A375 and A375-M6 cultures
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31 were mixed with Tris-Glycine SDS Native Sample Buffer (Invitrogen), electrophoresed through
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33 10 % Novex Zymogram Gelatin Gels (Invitrogen) and developed according to the manufacturer's
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35 instructions. The bands containing gelatinolytic activity of MMP2 and MMP9 appeared transparent
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37 and were evident in the otherwise homogeneous blue gel. Bands were quantified using ImageJ
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39 software.
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45 **Statistical analysis**

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47 Statistical analyses of the data were performed using one-way ANOVA, $P \leq 0.05$ was considered
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49 to be a statistically significant difference, and $P \leq 0.01$ was considered to be a very significant
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51 difference.
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RESULTS

Effect of senescence on SASP-related factors produced by MRC5 fibroblasts

MRC5 fibroblasts, either at low (young) or high (sen) PDL levels, were analyzed for the expression of a group of genes related to SASP. Figure 1A shows the results of Real Time PCR experiments for IL1 α ; IL1 β ; chemokine TGF β ; proteases MMP2 and uPA; and receptors uPAR, TGF β -R2, and CXCR4. The expression of all reported genes, except for TGF β -R2, resulted significantly increased in sen MRC5. IL-1 α was more than seven-fold upregulated in sen versus young MRC5 fibroblasts. A striking upregulation of CXCR4, with an approximately four-fold increase of UPA and TGF β , and two-fold uPAR mRNA levels were measured in sen compared to young fibroblasts.

Effect of chronic resveratrol treatment on SASP-related factors produced by MRC5 fibroblasts

Pre-senescent MRC5 fibroblast cultures (PDL>45) were treated for 5 weeks for replicative senescence (see methods), without (sen) and with (senR5) 5 μ M resveratrol concentration (17,18).

Figure 1B shows the results of Real Time PCR experiments on the expression of SASP-related interleukins IL1 α , IL1 β , IL6 and IL8; chemokine TGF β ; proteases MMP3, MMP2 and uPA; and receptors uPAR, IL6R, IGF-1R, TGF β -R2, and CXCR4. In treated MRC5 fibroblasts (senR5), IL1 α , IL1 β , IL6 and IL8 expression levels were reduced to 60%, 36%, 54% and 95% of the basal levels (untreated cultures, sen), respectively. Gene expression levels of matrix metallo-proteinases MMP2 and MMP3 and serine protease uPA were found decreased to approximately 60%, which was accompanied by a reduction in the mRNA levels of the uPAR, IL6R, IGF-1R, TGF β -R2 receptors, with the strongest effect on CXCR4 expression. Zymogram analysis (Figure 1C) of 24 h CM, collected after resveratrol treatment (senR5), revealed a 30% reduction in the MMP2 active protein secretion, while MMP9 was almost unaffected compared to control CM from untreated cultures (sen). Western blot analyses (Figure 1D) of senR5 cell extracts, compared to sen, showed

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3 reduced protein levels of NF- κ B, uPAR (to approximately 60%, both), and TGF β -R2 (to
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5 approximately 85%).
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9 **Effect of chronic resveratrol pre-treatment on MRC5 activation by TGF β**

10 SenR5 and sen fibroblast cultures were incubated for 24 h with or without 10 ng/mL TGF β 1 (see
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12 methods); then, cell extracts underwent to western blot analyses. For zymogram analyses, CM were
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14 obtained in parallel cell cultures after TGF β 1 treatment, for 24 h in DMEM plus 2% FBS, as
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16 reported in the methods section.
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21 Figure 2A shows that TGF β 1 treatment of senescent cultures (sen+TGF β 1) induced a four-fold
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23 increase in the cellular alpha-smooth actin (alpha-SMA) protein level. Resveratrol pre-treatment
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25 was effective in reducing the sen fibroblast activation by TGF β 1. In fact, in senR5+TGF β 1 cell
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27 extracts, the alpha-SMA protein concentration decreased the levels to near those of unstimulated
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29 cultures (sen).
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32 Accordingly, zymogram analysis (Figure 2B) of CM after TGF β 1 treatment showed a
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34 significant increase in the MMP2 activity (sen+TGF β 1 versus sen). R5 pre-incubation of sen
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36 cultures revealed a significant reduction in the MMP2 activity, both with (CM senR5+TGF β 1
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38 versus CM sen+TGF β 1) and without (CM senR5 versus CM sen) stimulation by TGF β 1. Moreover,
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40 R5 pre-treatment of TGF β 1 stimulated sen cultures induced CM levels of MMP2 activity that were
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42 even lower than those of sen unstimulated cells (CM senR5+TGF β 1 versus CM sen). By contrast,
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44 MMP9 activity did not increase with TGF β 1 treatment and was almost unaffected by R5 pre-
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46 treatment.
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52 **Effect of CM from MRC5 fibroblasts on the invasive activity of A375 and A375-M6**
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54 **melanoma cells** Conditioned media obtained from low PDL (<30) MRC5 cultures (CM young),
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56 together with media obtained from high PDL MRC5 cultures, either untreated (CM sen) or
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58 chronically pre-treated with 5 μ M resveratrol (CM senR5), were used for the experiments with
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3 A375 (Figure 3, A and B) and A375-M6 (Figure 3, C and D) melanoma cells. Additionally, fresh
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5 DMEM plus 2% FBS (DMEM) was used as a reference condition.
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7 Chemoinvasion experiments were performed as described in the methods section. Briefly,
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9 melanoma cell suspensions were placed in upper chambers of Matrigel-coated Transwell cell
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11 culture chambers and different conditioned media were added to the lower chambers. After 6 h
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13 incubation at 37°C, cells that had passed through the lower surface of the filter were photographed
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15 (see supplementary Figure 1) and counted; then, results were reported as histograms. CM from sen
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17 MRC5 cultures stimulated the invasion of A375-M6 cells (panel C), with there were approximately
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19 70% and 50% increases in the migrated cell numbers compared to those that migrated toward
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21 DMEM, and toward CM young, respectively. CM from senR5 fibroblasts reduced the invasiveness
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23 of melanoma A375-M6 cells more than 40% compared to cells that migrated towards CM sen,
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25 reaching the levels obtained with young fibroblast CM. Additionally A375 cell migration (panel A)
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27 was slightly, but significantly increased by CM from sen fibroblasts, and when CM from senR5
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29 fibroblasts was placed in the lower chamber, the number of migrated cells was decreased to values
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31 approximately 50% of the values measured towards either unconditioned DMEM or CM young. In
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33 spontaneous invasion experiments (see photos in Supplementary Figure 1), subconfluent A375 and
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35 A375-M6 cultures were incubated for 48 h with CMs obtained from young, sen and senR5
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37 fibroblast cultures. Melanoma cells were then collected, counted, suspended in DMEM medium
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39 containing 2% FBS and seeded in the upper chamber of Matrigel-coated Transwell, as described
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41 above, except for the presence of standard DMEM plus 2% FBS in all lower chambers. After 6 h
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43 incubation at 37°C, the spontaneous invasive activity of A375-M6 (panel D) melanoma cells after
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45 incubation with CM sen resulted in increases of 30% and 40% compared to unconditioned DMEM
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47 and to young fibroblast CM, respectively. The pre-incubation with CM senR5 was capable of
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49 reducing the A375-M6 spontaneous invasive activity to approximately 50%, compared to
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51 incubation with CM sen, and to levels slightly lower than those that were measured after incubation
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3 with CM young. No significant differences were measured in the spontaneous invasive activity of
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5 A375 melanoma cells (panel B).
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10 **Effects of CM from MRC5 fibroblasts on the growth and EMT phenotype of A375 and A375-**
11 **M6 melanoma cells.**
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14 In these experiments, A375 and A375-M6 cultures were maintained for 72 h with the above
15 described MRC5 conditioned media, namely CM sen, CM senR5, and unconditioned DMEM.
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17 Melanoma cells were counted after 24, 48 and 72 h of incubation. All counted cells were viable
18 (according to Trypan blue exclusion test, data not shown). At 48 h, cells from parallel cell cultures
19 were collected, and used to obtain cell protein extracts. Figure 4 shows that medium produced by
20 sen fibroblasts was effective in promoting the growth of A375 melanoma cells (Figure 4A), and
21 there was a 40% increase in the cell count at 48 h incubation. This was not observed in A375-M6
22 cells (Figure 4C), which, as expected, showed a higher growth rate compared to A375 cultures, and
23 were found confluent at 72 h in either DMEM or sen CM conditions. Incubation of both A375 and
24 A375-M6 cells with CM from senR5 fibroblasts produced a time-dependent inhibition of tumor cell
25 proliferation, reaching 30% of the A375-M6 cell number reduction at 48 h, compared to both
26 incubations with CM sen and with unconditioned DMEM. Incubation with senR5 CM showed a
27 more pronounced effect on A375 cell growth, and there was a 50% reduction compared to basal
28 DMEM conditions, and 60% compared to sen CM, both at 48 and 72 h.
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45 Western blot analyses (Figure 4, B and D) show that incubation with CM sen, compared to
46 incubation with DMEM, produced an approximately 30 % increase in the p38 phosphorylation in
47 A375-M6 cells, and almost four-fold increase in the P-Erk p42 in both cell lines. In A375 cells,
48 nearly 40% increase in the P-erk p44 was measured, and it was approximately four-fold increased
49 upon sen CM incubation of A375-M6, compared to incubation with DMEM. All phosphorylated
50 forms of p38 and Erk1/2 were negatively modulated in both A375 and A375-M6 melanoma cells
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3 that were incubated with CM senR5, and there was a striking reduction of P-p38, reaching levels
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5 that were 60% lower than incubation with unconditioned DMEM.
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8 Figure 5 shows the results of m-RNA expression for a panel of EMT-related genes, measured by
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10 RT PCR in either A375 (panel A) or A375-M6 (panel D) cells after 48 h incubation with different
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12 CMs, as described above and reported as a percentage of results after incubation with unconditioned
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14 DMEM (indicated by the dot line at 100%). A significant increase in SDF1/CXCR4, and vimentin
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16 expression levels was measured in A375 melanoma cells that were incubated with CM sen
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18 compared to unconditioned DMEM incubation. In addition to the above reported genes, expression
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20 levels of uPA/uPAR and N-cad were significantly reduced by senR5 CM incubation compared to
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22 both CM sen and unconditioned DMEM conditions. Among the analyzed genes, a significant
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24 increase in the CXCR4, MMP2, UPA/uPAR and N-cad expression levels was measured in A375-
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26 M6 melanoma cells incubated with CM sen compared to unconditioned DMEM incubation. By
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28 contrast, incubation of A375-M6 with CM senR5 determined a reduced expression of all genes
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30 compared to levels measured after incubation with CM sen. Expression of SDF1/CXCR4,
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32 uPA/uPAR, N-cad, Vimentin (VIM) and MMP9 was markedly reduced to the same or even lower
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34 levels than after incubation with unconditioned DMEM. Accordingly, western blot analyses of
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36 melanoma cell extracts (panel B and E) for receptor proteins CXCR4 and uPAR, and for N-cad,
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38 confirmed the RT PCR results. In addition, uPAR and N-cad protein levels were strongly reduced
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40 by CM senR5 compared to CM sen and DMEM, while the effect on CXCR4 protein was less than
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42 anticipated from the RT PCR results.
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47 Twenty-four h-conditioned media samples produced by A375 and A375-M6 cultures, were
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49 collected after a 48-h pre-incubation with DMEM or CM from sen and senR5 fibroblasts and
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51 submitted to zymography (panel C and F) to detect MMP2 and MMP9 activity. No significant
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53 differences were measured in A375 melanoma cells (panel C). After pre-incubation with CM sen,
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55 only MMP2 activity was increased in CM produced by A375-M6 (panel F) melanoma cells,
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57 reaching values that were approximately 50% higher than the basal levels. Both gelatinolytic
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3 activities were reduced by pre-incubation with CM senR5, and with MMP2 reduced back to basal
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5 levels and MMP9 was reduced to lower than the basal levels.
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23 DISCUSSION

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25 The ability of senescent human fibroblasts to promote carcinogenesis has been described and
26 related to SASP factors in several papers by Campisi and co-workers (2,4-6,11,12, 20, 21). Cell
27 senescence is a consequence of telomeric dysfunction related to repeated cell division (22); in
28 addition, it is also a response to genotoxic stresses induced by anti-cancer therapeutic interventions,
29 such as X-irradiation or chemotherapy (23). Recently, induction of senescence has been employed
30 as a therapeutic approach in various tumours, including melanoma (24). At the same time, adverse
31 effects produced by cellular senescence were measured and ascribed to SASP (25). Thus, in
32 developing anti-cancer treatments, it might be necessary to develop adjuvant therapies targeting the
33 cancer-promoting SASP, and at the same time maintaining the beneficial features of cell senescence
34 (26,27).
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47 We have previously reported that, in senescent MRC5 fibroblasts, the R5 treatment protocol
48 induces a reduction in the expression levels and release of inflammatory IL6 and IL8, as well as of
49 growth factors GRO α and VEGF (18), which are responsible for inducing EMT and proliferation in
50 both epithelial and endothelial cells (4). In this study, our senescent fibroblast model was validated
51 by results obtained comparing SASP expression levels in young versus senescent MRC5 cultures.
52 Interestingly, IL-1 α , reported to be a general regulator of senescence-associated IL-6/IL-8 secretion
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3 (28), has been found more than 7-fold upregulated in senescent fibroblasts along with the increase
4 in MMP2 and TGF β mRNA levels (four-fold increased), and a striking upregulation of CXCR4.
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7 We confirmed those previous published data (18) and evaluated further SASP factors that are
8 potentially related to the pro-tumoral activity of our senescence model. These included metallo-
9 proteinases and uPA, enzymes that are involved in altering the ECM, as well as surface receptor
10 molecules, such as uPAR, CXCR4 and TGF β -R2. In this regard, the uPA/uPAR system is known to
11 be involved in tumor-related processes, such as cell migration, proliferation and angiogenesis
12 (9,10,29), and the SDF1/CXCR4 axis is the major mechanism responsible for autocrine and
13 paracrine regulation of metastatic cancer features, which are primarily related to cell homing and
14 migration (8,30). The R5-induced decrease in mRNA expression levels was confirmed by
15 zymogram analyses for MMP2 activity in CM produced by R5-treated senescent fibroblasts and by
16 cellular protein levels of receptors uPAR and TGF β -R2. Moreover, we observed a significant
17 decrease in the cellular NF- κ B protein level, a transcription factor that has yet to be reported to play
18 a pivotal role in inducing SASP (31). Our chronic resveratrol treatment inhibited the activation of
19 MRC5 to myofibroblast when exposed to TGF β 1, which was also reported by Fagone et al. (32) in
20 *ex vivo* human lung fibroblasts and by Olson et al. (33) in cardiac fibroblasts that were exposed to
21 angiotensin II. Myofibroblasts secrete and activate TGF β , establishing a self-sustained feedback,
22 and autocrine TGF β - signaling has been shown to drive myofibroblast differentiation during tumor
23 progression (34). In this context, the inhibition of the TGF β 1 effect, together with the lower
24 expression of TGF β and TGF β -R2 mediated by R5 treatment of MRC5 senescent fibroblasts, might
25 confer protection against signaling mediated stroma activation. Upon TGF β 1-stimulation, MMP2
26 production and release in the medium by senescent MRC5 was counteracted by R5.
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51 Taken together, these findings indicate that R5 treatment was effective in suppressing the major
52 SASP components in our MRC5 senescence model, even in a TGF β -activated microenvironment.
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54 Metastatic melanoma is the most aggressive form of skin cancer, and novel therapeutic
55 approaches have been developed with inhibitors of the RAF/MEK pathways co-targeted strategies,
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3 to bypass the frequent development of chemo-resistance (35). In recent years, significant attention
4 has been placed on nutraceuticals, due to their disease modifying activities (36). Recently,
5 preclinical studies on phytochemicals, both alone and in combination with traditional cytotoxic and
6 targeted therapies, yielded promising results on melanoma treatment (reviewed by Strickland et al.,
7 37). Additionally, resveratrol showed a role in counteracting multidrug resistance, by
8 simultaneously acting on diverse mechanisms, that are relevant in both cancer prevention and
9 treatment (16). In the present paper, to evaluate the effects of R5 treatment on the tumorigenic
10 activity of senescent MRC5 secretion, conditioned media were tested on **two human melanoma cell**
11 **lines: A375, a cell line carrying BRAF mutation (38) that is characterized by a low metastatic**
12 **potential, and A375-M6 cells, a murine lung metastatic cell line derived from A375.** Functional
13 assays on the invasive properties of melanoma cells revealed a pro-tumoral effect of CM produced
14 by senescent MRC5, compared with both unconditioned medium and CM produced by low PDL,
15 young MRC5 cultures. An increase in the invasiveness of **both melanoma cell lines** was measured
16 when senescent CM was used as a chemo-attractant. This chemo-attractant **was less effective for**
17 **A375 melanoma, than for A375-M6 cells** when it was used in 48 h pre-treatment of cancer cells
18 before the invasion test (**reported as spontaneous invasion test**). **A375 and** A375-M6 cells have been
19 analysed for mRNA expression of genes that directly or indirectly could be related to the pro-
20 tumorigenic effect of CM from senescent MRC5. **We** found a significant increase in the expression
21 of the EMT marker N-cad, of CXCR4, of uPA/uPAR system, and gelatinase MMP2, and all of
22 these could be associated with the increase in melanoma cell invasiveness (39-42). **The** R5
23 treatment protocol was capable of reducing the deleterious effects induced by senescent CM on
24 **A375 and A375-M6** malignant features. Specifically, the treatment reduced the SDF1/CXCR4 axis,
25 MMP2 protease activity, and uPA/uPAR system. In addition, it reduced EMT markers N-cad and
26 Vim. **All of these** molecular changes most likely underlie the concomitant reduction in melanoma
27 invasiveness.
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3 The mitogen-activated protein kinase (MAPK) signaling pathway is associated with cell
4 differentiation, proliferation, and survival. Four MAPK cascades have been identified, ERK1/2, c-
5 Jun N-Terminal kinase (JNK), p38, and ERK5 cascades. In the present work, we found an increase
6 in phosphorylation of both p38 and ERK1/2 after 48 h of incubation of both A375 and A375-M6
7 melanoma cells with CM produced by senescent MRC5 fibroblasts, which further confirmed the
8 pro-tumoral activity of SASP. Conversely, the incubation with CM from R5-treated senescent cells
9 was effective in reducing the phosphorylation levels of both proteins. The negative modulation of
10 P-ERK1/2 could also account for the observed concomitant down-regulation of uPAR (43,44).
11 Similar results were recently reported by Liu et al (45) who used simvastatin to reduce senescent
12 fibroblast SASP deleterious effects on breast cancer cell proliferation. Accordingly, the
13 proliferation rate of melanoma cells was reduced by CM from R5-treated senescent cells. Given the
14 correlation between the metastatic potential and proliferative activity of A375 melanoma cells (46),
15 our results suggest that the regulation of SASP by R5 treatment might be useful for controlling
16 A375 tumor progression, through inhibiting of A375 proliferation, together with the substantial
17 decrease in EMT markers, SDF1/CXCR4 and UPA/uPAR systems.

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36 Overall, senR5 CM had slightly different effects on the tumoral phenotype of A375 and A375-
37 M6 melanoma cell lines. It was more efficacious in decreasing the growth-related phenotypes of
38 A375 melanoma and it was not very effective against cell spontaneous invasion after treatment,
39 which is probably due to the low basal metastatic potential of A375, but their migration toward
40 senR5 medium was significantly reduced, confirming the anti-tumoral effect of R5 pre-treatment on
41 senescent fibroblast CM. However, senR5 CM incubation was capable of more efficiently reducing
42 the spontaneous invasive activity of A375-M6 metastatic melanoma cells, indicating a possible
43 effect in reducing their metastatic potential, which was also demonstrated by the decrease in MMPs
44 gene expression and activities.

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3 We can conclude that chronic resveratrol treatment might be a powerful strategy in
4 counteracting the “dark side” of senescence without affecting the tumour suppressive mechanisms.
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7 In fact, we previously showed that R5 treatment did not extend the MRC5 lifespan (17) and did not
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9 modify the p16/INK4a expression (18) of senescent treated fibroblasts. The protective, anti-tumoral
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11 activity of conditioned media from R5-treated senescent MRC5 fibroblasts might be due to a
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13 decrease in a wide-range of detrimental SASP factors.
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16 This innovative experimental approach supports the development of preventive and anti-cancer
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18 treatments that target the stromal cell phenotype, which can indirectly modulate the neoplastic
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20 features of melanoma cells.
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26
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28
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32 **CONFLICT OF INTEREST**

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34 The authors declare that they have no conflict of interest to report
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Figure Legends

Figure 1. MRC5 senescent phenotype and effect of resveratrol treatment on gene expression and secretion of MRC5 SASP-related factors. (A) MRC5 fibroblasts, either at low (young) or high (sen) PDL levels, were analyzed for the expression of a group of genes related to SASP by Quantitative RT PCR, using GAPDH as the housekeeping gene. Histograms represent sen culture values normalized to young cultures (assumed as value 100% and indicated as the dot line) according to $2^{-\Delta\Delta CT}$ method. * shows statistical significance ($p < 0.05$), ** show a very statistical significance ($p < 0.01$) compared to young. (B) Pre-senescent MRC5 fibroblast cultures were treated for 5 weeks in the absence (sen) or in the presence (senR5) of 5 μ M resveratrol and analyzed by quantitative RT PCR for expression of indicated SASP-related genes. Data were reported as senR5 mRNA values normalized to untreated culture results (sen, assumed as value 100% and indicated as the dot line) according to $2^{-\Delta\Delta CT}$ method and using GAPDH as the housekeeping gene. (C) Zymogram analysis of MRC5 conditioned media (CM). After 5 weeks of treatment, sen and senR5 cultures were incubated for 24 h in DMEM in order to obtain CM. MMP-2 and MMP-9 activities were detected by gelatin zymography analysis as transparent bands (shown on the left) and quantified by ImageJ; histograms represent MMP-2 and MMP-9 activity measured in CM from R5 treated senescent fibroblasts (senR5) and referred as a percentage of values obtained in CM from untreated cultures (sen, dot line). Results represent the mean of three different experiments \pm SD. (D) Western Blot analyses of NF-kB, uPAR, TGF β -R2 protein amounts in sen and senR5. Bands (shown on the left) were quantified by ImageJ; histograms represent the mean of the protein concentration detected in

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3 three different experiments \pm SD. Data were normalized to untreated cultures (sen, assumed as
4 value 100% and indicated as the dot line) and reported as a percentage. Tubulin was used as a
5 loading control. In B,C and D * shows statistical significance ($p < 0.05$), ** show a very statistical
6 significance ($p < 0.01$) between senR5 and sen.
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11 **Figure 2.** Resveratrol chronic pre-treatment inhibits MRC5 fibroblast activation by TGF β 1. Pre-
12 senescent MRC5 fibroblasts were treated for 5 weeks without (sen) or with (senR5) 5 μ M
13 resveratrol, then were incubated for 24 h with 10 ng/mL TGF β 1. (A) Western Blot analysis of α -
14 SMA protein concentration. Bands (shown on the left) were quantified by ImageJ; histograms
15 represent the mean of the protein levels detected in three different experiments \pm SD. Results,
16 reported as a percentage, were normalized to values obtained in sen, assumed as 100%. Tubulin
17 was used as loading control. (B) Zymogram analysis of 24 h CM harvested in sen and senR5 after
18 TGF β 1 stimulation. Gelatinolytic activity was detected by zymogram analysis as transparent bands
19 (shown on the left) and quantified by ImageJ; histograms show MMP-2 and MMP-9 activity in CM
20 from sen and sen R5 without or with TGF β 1 stimulation. Results, reported as a percentage,
21 represent the mean of three different experiments \pm SD and are normalized to data obtained in CM
22 from sen cultures that were not submitted to TGF β 1 stimulation (assumed as value 100%). * shows
23 statistical significance ($p < 0.05$), between sen+TGF β 1 and senR5+TGF β 1, # shows statistical
24 significance ($p < 0.05$) compared to sen.
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43 **Figure 3.** Effect of CM from MRC5 on A375 and A375-M6 cell invasion. 24h-CM from young
44 fibroblasts (CM young) and from senescent cultures, either untreated (CM sen) or treated (CM
45 senR5) for 5 weeks with 5 μ M resveratrol were used in invasion experiments. Invasion test was
46 performed with 2×10^4 A375 and 10^4 A375-M6 cells, for 6 hours at 37°C. (A,C) Matrigel
47 chemoinvasion. A375 (A) and A375-M6 (C) melanoma cells were placed in the upper well while
48 CM from young, sen and senR5 fibroblasts were placed in the lower one. Fresh DMEM was used as
49 a reference condition. Histograms represent the mean of three different experiments \pm SD, results
50 are reported as a percentage of the migrated cells toward CM young, CM sen or CM senR5,
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3 compared to those **that** migrated toward fresh DMEM (assumed as value 100%).*shows statistical
4 significance (p<0.05) compared to CM sen. # shows statistical significance (p<0.05) compared to
5 unconditioned DMEM. **(B, D)** Spontaneous invasion. **A375 (B) and A375-M6 (D)** melanoma cells
6 were incubated for 48 h with CM young, CM sen, CM senR5 or unconditioned DMEM (used as a
7 reference condition). After incubation, melanoma cells were suspended in fresh DMEM that was
8 placed also in the lower well. Histograms represent the mean of three different experiments \pm SD,
9 results are reported as a percentage of the migrated cells after pre-incubation with MRC5 CM
10 compared to those **that** migrated after pre-incubation with DMEM (assumed as value 100%). *
11 shows statistical significance (p<0.05) compared to pre-incubation with CM sen. # shows statistical
12 significance (p<0.05) compared to pre-incubation with DMEM. **Representative photographs of**
13 **invasion experiments are shown in Supplementary Figure 1.**

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28 **Figure 4.** Effect of CM from MRC5 on the cell growth **and kinase phosphorylation of A375 and**
29 **A375-M6. Melanoma** cells were seeded (2×10^4 per cm^2) and incubated with CM from sen, senR5
30 fibroblasts or unconditioned DMEM. After 24, 48 **and 72 h of** incubation with CM, **A375 (A) and**
31 **A375-M6 (C)** melanoma cells were counted in a Bürker chamber. Cell viability was assessed by the
32 Trypan Blue exclusion test. Results were expressed as the mean of three different experiments \pm
33 SD. * shows statistical significance (p<0.05) compared to incubation with CM from sen fibroblasts,
34 # shows statistical significance (p<0.05) compared to incubation with DMEM. Western Blot
35 analyses of p38, P-p38, erk42/44 and P-erk 42/44 protein concentration in **A375 (B) and A375-M6**
36 **(D)** cells after 48h incubation with CM from sen and senR5 fibroblasts or with unconditioned
37 DMEM. Tubulin was used as a loading control. Bands (shown on the left) were quantified by
38 ImageJ; histograms represent the mean of protein concentrations of three different experiments \pm
39 SD. Results, reported as a percentage, were normalized to values obtained after incubation with
40 unconditioned DMEM (assumed as value 100%, dot line). * shows statistical significance (p<0.05)
41 compared to incubation with CM from sen fibroblasts. # shows statistical significance (p<0.05)
42 compared to incubation with DMEM.

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3 **Figure 5.** Effect of CM senR5 on EMT phenotype of **A375** and A375-M6 melanoma cells. **Cell**
4 cultures were incubated for 48 h with CM from sen, senR5 fibroblasts or unconditioned DMEM.
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7 **(A, D)** Quantitative RT PCR of EMT-related gene expression. Results (shown in histograms) are
8 reported as a percentage of values obtained according to $2^{-\Delta\Delta CT}$ method in **A375 (A)** and A375-M6
9 **(D) melanoma cells** after incubation with unconditioned DMEM (assumed as value 100%, dot line)
10 and represent the mean of three different experiments \pm SD. GAPDH was used as the housekeeping
11 gene. * shows statistical significance ($p < 0.05$) compared to incubation with CM from sen
12 fibroblasts. # shows statistical significance ($p < 0.05$) compared to incubation with DMEM. **(B,E)**
13 Western Blot analyses of CXCR4, uPAR, and N-cad protein concentration in **A375 (B)** and **A375-**
14 **M6 (E)**. Bands (shown on the top) were quantified by ImageJ; histograms represent the mean of the
15 protein amount of three different experiments \pm SD. Results were reported as a percentage and were
16 normalized to values obtained after melanoma cell incubation with unconditioned DMEM (assumed
17 as value 100%, dot line). Tubulin was used as a loading control. **(C,F)** Zymogram analyses of 24 h
18 CM harvested in **A375 (C)** and **A375-M6 (F)** after 48 h pre-incubation with CM from sen, senR5
19 fibroblasts or unconditioned DMEM. MMP-2 and MMP-9 activities were detected by gelatin
20 zymography analysis and appeared as transparent bands (shown on the top). ImageJ was used for
21 quantification. Histograms represent the mean of the gelatinolytic activity of **melanoma CM**
22 **measured in** three different experiments \pm SD. Results are reported as a percentage of values
23 obtained after pre-incubation with unconditioned DMEM (assumed as value 100%, dot line). *
24 shows statistical significance ($p < 0.05$) and ** a very statistical significance ($p < 0.01$), compared to
25 incubation with CM from sen fibroblasts. # shows statistical significance ($p < 0.05$) compared to
26 incubation with DMEM.
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51 **Supplementary Figure 1**

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55 **Representative photographs of the invasive activity of A375 and A375-M6 melanoma cells.**
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57 **Conditioned media were collected from young fibroblasts (CM young) and from senescent cultures,**
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3 either untreated (CM sen) or treated (CM senR5) for 5 weeks with 5 μ M resveratrol and were used
4 in invasion experiments. Matrigel chemoinvasion. Melanoma cells were placed in the upper well
5 while unconditioned DMEM, CM young, CM sen and CM senR5 were placed in the lower one.
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7 Spontaneous invasion. Melanoma cells were incubated for 48 h with unconditioned DMEM, CM
8 young, CM sen and CM senR5 then were suspended in fresh DMEM, that was also placed in the
9 lower well. Photographs show representative fields (10x) of cells that after 6 hours at 37°C had
10 passed the Matrigel-coated filters, after fixing and staining by the Diff-Quick method.
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For Peer Review

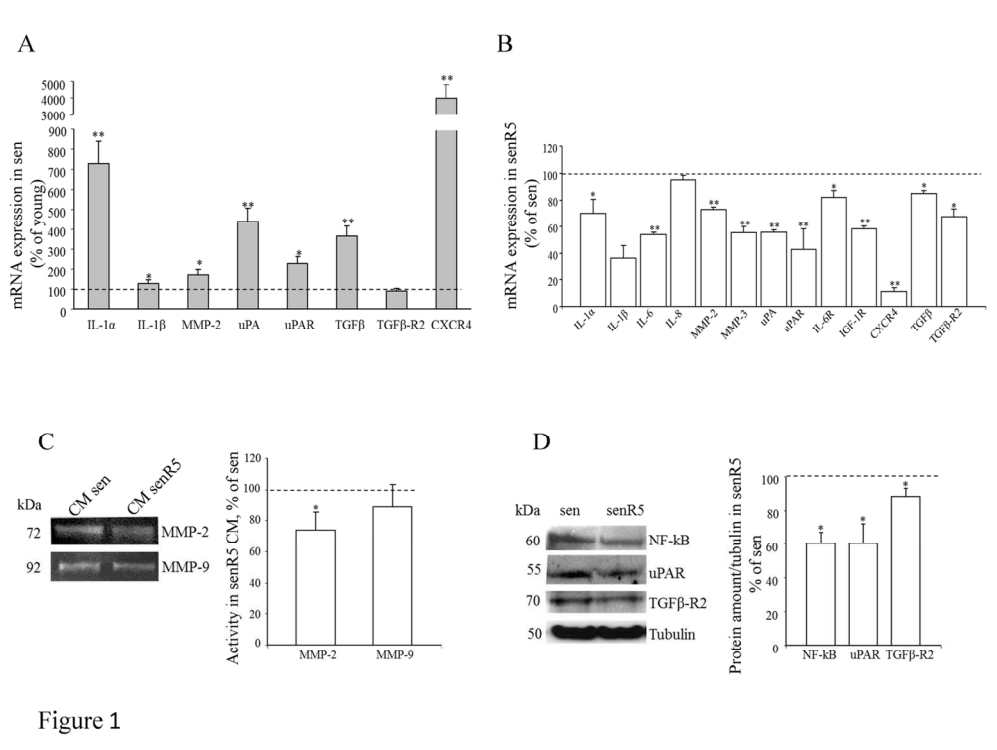
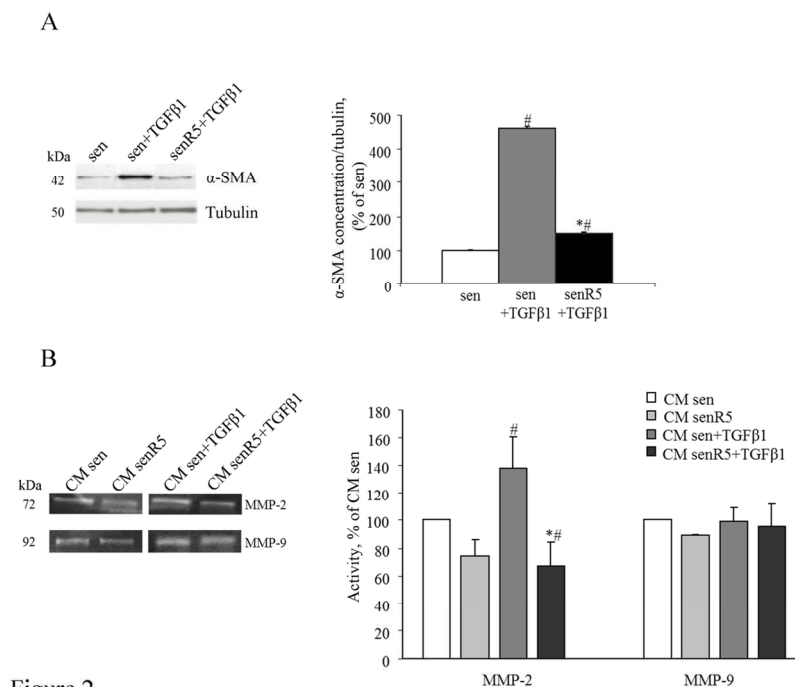


Figure 1

Figure 1. MRC5 senescent phenotype and effect of resveratrol treatment on gene expression and secretion of MRC5 SASP-related factors. (A) MRC5 fibroblasts, either at low (young) or high (sen) PDL levels, were analyzed for the expression of a group of genes related to SASP by Quantitative RT PCR, using GAPDH as the housekeeping gene. Histograms represent sen culture values normalized to young cultures (assumed as value 100% and indicated as the dot line) according to 2- $\Delta\Delta$ CT method. * shows statistical significance ($p < 0.05$), ** show a very statistical significance ($p < 0.01$) compared to young. (B) Pre-senescent MRC5 fibroblast cultures were treated for 5 weeks in the absence (sen) or in the presence (senR5) of 5 μ M resveratrol and analyzed by quantitative RT PCR for expression of indicated SASP-related genes. Data were reported as senR5 mRNA values normalized to untreated culture results (sen, assumed as value 100% and indicated as the dot line) according to 2- $\Delta\Delta$ CT method and using GAPDH as the housekeeping gene. (C) Zymogram analysis of MRC5 conditioned media (CM). After 5 weeks of treatment, sen and senR5 cultures were incubated for 24 h in DMEM in order to obtain CM. MMP-2 and MMP-9 activities were detected by gelatin zymography analysis as transparent bands (shown on the left) and quantified by ImageJ; histograms represent MMP-2 and MMP-9 activity measured in CM from R5 treated senescent fibroblasts (senR5) and referred as a percentage of values obtained in CM from untreated cultures (sen, dot line). Results represent the mean of three different experiments \pm SD. (D) Western Blot analyses of NF-kB, uPAR, TGF β -R2 protein amounts in sen and senR5. Bands (shown on the left) were quantified by ImageJ; histograms represent the mean of the protein concentration detected in three different experiments \pm SD. Data were normalized to untreated cultures (sen, assumed as value 100% and indicated as the dot line) and reported as a percentage. Tubulin was used as a loading control. In B,C and D * shows statistical significance ($p < 0.05$), ** show a very statistical significance ($p < 0.01$) between senR5 and sen.

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Figure 2. Resveratrol chronic pre-treatment inhibits MRC5 fibroblast activation by TGF β 1. Pre-senescent MRC5 fibroblasts were treated for 5 weeks without (sen) or with (senR5) 5 μ M resveratrol, then were incubated for 24 h with 10 ng/mL TGF β 1. (A) Western Blot analysis of α -SMA protein concentration. Bands (shown on the left) were quantified by ImageJ; histograms represent the mean of the protein levels detected in three different experiments \pm SD. Results, reported as a percentage, were normalized to values obtained in sen, assumed as 100%. Tubulin was used as loading control. (B) Zymogram analysis of 24 h CM harvested in sen and senR5 after TGF β 1 stimulation. Gelatinolytic activity was detected by zymogram analysis as transparent bands (shown on the left) and quantified by ImageJ; histograms show MMP-2 and MMP-9 activity in CM from sen and sen R5 without or with TGF β 1 stimulation. Results, reported as a percentage, represent the mean of three different experiments \pm SD and are normalized to data obtained in CM from sen cultures that were not submitted to TGF β 1 stimulation (assumed as value 100%). * shows statistical significance ($p < 0.05$), between sen+TGF β 1 and senR5+TGF β 1, # shows statistical significance ($p < 0.05$) compared to sen.

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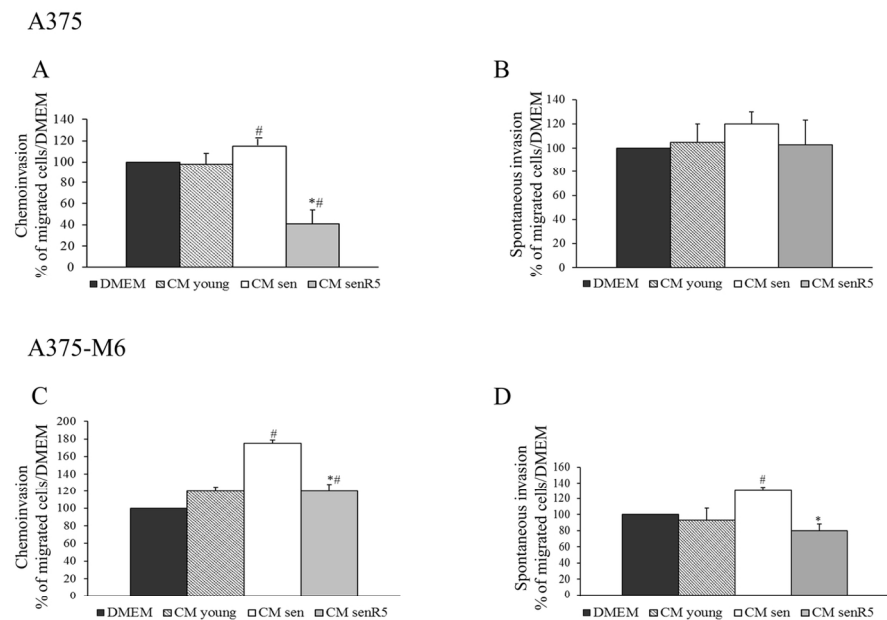


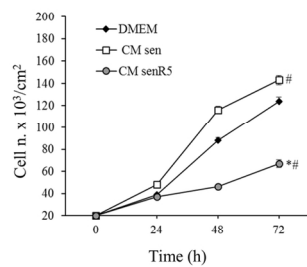
Figure 3

Figure 3. Effect of CM from MRC5 on A375 and A375-M6 cell invasion. 24h-CM from young fibroblasts (CM young) and from senescent cultures, either untreated (CM sen) or treated (CM senR5) for 5 weeks with 5µM resveratrol were used in invasion experiments. Invasion test was performed with 2 x 10⁴ A375 and 10⁴ A375-M6 cells, for 6 hours at 37°C. (A,C) Matrigel chemoinvasion. A375 (A) and A375-M6 (C) melanoma cells were placed in the upper well while CM from young, sen and senR5 fibroblasts were placed in the lower one. Fresh DMEM was used as a reference condition. Histograms represent the mean of three different experiments ± SD, results are reported as a percentage of the migrated cells toward CM young, CM sen or CM senR5, compared to those that migrated toward fresh DMEM (assumed as value 100%). *shows statistical significance (p<0.05) compared to CM sen. # shows statistical significance (p<0.05) compared to unconditioned DMEM. (B, D) Spontaneous invasion. A375 (B) and A375-M6 (D) melanoma cells were incubated for 48 h with CM young, CM sen, CM senR5 or unconditioned DMEM (used as a reference condition). After incubation, melanoma cells were suspended in fresh DMEM that was placed also in the lower well. Histograms represent the mean of three different experiments ± SD, results are reported as a percentage of the migrated cells after pre-incubation with MRC5 CM compared to those that migrated after pre-incubation with DMEM (assumed as value 100%). * shows statistical significance (p<0.05) compared to pre-incubation with CM sen. # shows statistical significance (p<0.05) compared to pre-incubation with DMEM. Representative photographs of invasion experiments are shown in Supplementary Figure 1.

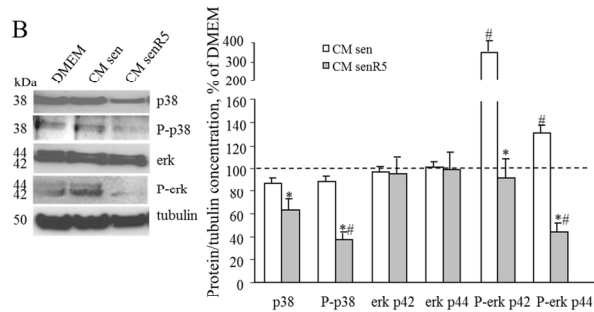
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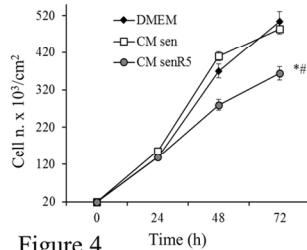


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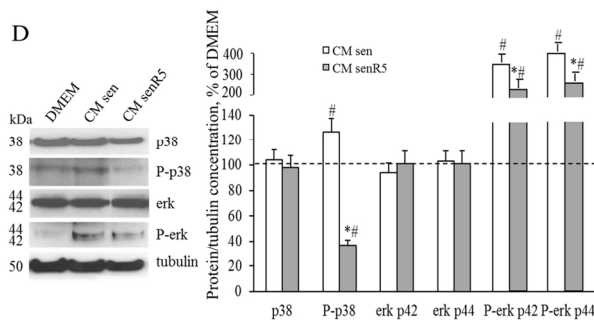


Figure 4

Figure 4. Effect of CM from MRC5 on the cell growth and kinase phosphorylation of A375 and A375-M6. Melanoma cells were seeded (2×10^4 per cm^2) and incubated with CM from sen, senR5 fibroblasts or unconditioned DMEM. After 24, 48 and 72 h of incubation with CM, A375 (A) and A375-M6 (C) melanoma cells were counted in a Bürker chamber. Cell viability was assessed by the Trypan Blue exclusion test. Results were expressed as the mean of three different experiments \pm SD. * shows statistical significance ($p < 0.05$) compared to incubation with CM from sen fibroblasts, # shows statistical significance ($p < 0.05$) compared to incubation with DMEM. Western Blot analyses of p38, P-p38, erk42/44 and P-erk 42/44 protein concentration in A375 (B) and A375-M6 (D) cells after 48h incubation with CM from sen and senR5 fibroblasts or with unconditioned DMEM. Tubulin was used as a loading control. Bands (shown on the left) were quantified by ImageJ; histograms represent the mean of protein concentrations of three different experiments \pm SD. Results, reported as a percentage, were normalized to values obtained after incubation with unconditioned DMEM (assumed as value 100%, dot line). * shows statistical significance ($p < 0.05$) compared to incubation with CM from sen fibroblasts. # shows statistical significance ($p < 0.05$) compared to incubation with DMEM.

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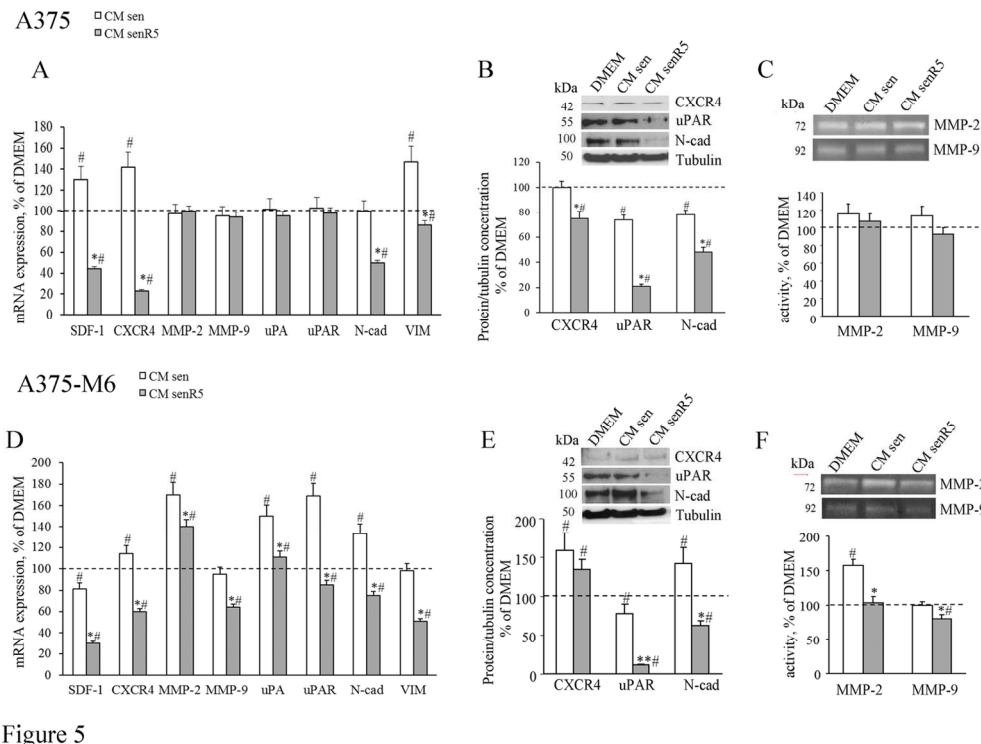


Figure 5

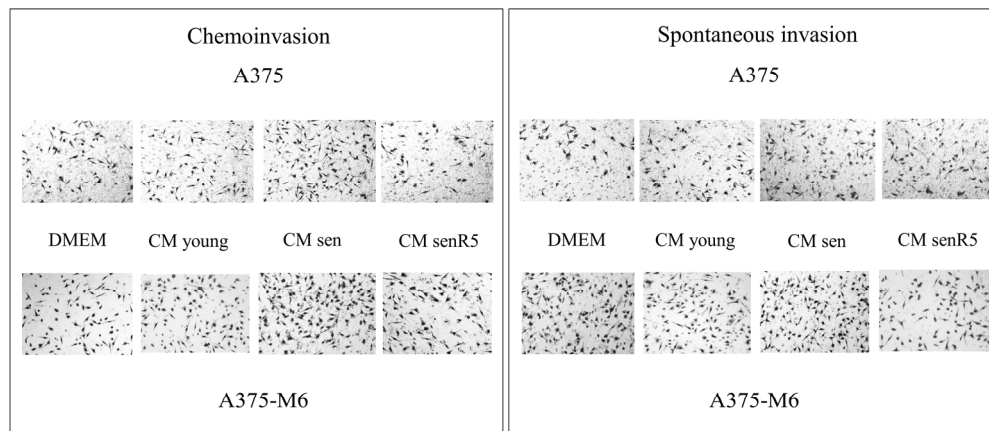
Figure 5. Effect of CM senR5 on EMT phenotype of A375 and A375-M6 melanoma cells. Cell cultures were incubated for 48 h with CM from sen, senR5 fibroblasts or unconditioned DMEM. (A, D) Quantitative RT PCR of EMT-related gene expression. Results (shown in histograms) are reported as a percentage of values obtained according to 2- $\Delta\Delta$ CT method in A375 (A) and A375-M6 (D) melanoma cells after incubation with unconditioned DMEM (assumed as value 100%, dot line) and represent the mean of three different experiments \pm SD. GAPDH was used as the housekeeping gene. * shows statistical significance ($p < 0.05$) compared to incubation with CM from sen fibroblasts. # shows statistical significance ($p < 0.05$) compared to incubation with DMEM. (B,E) Western Blot analyses of CXCR4, uPAR, and N-cad protein concentration in A375 (B) and A375-M6 (E). Bands (shown on the top) were quantified by ImageJ; histograms represent the mean of the protein amount of three different experiments \pm SD. Results were reported as a percentage and were normalized to values obtained after melanoma cell incubation with unconditioned DMEM (assumed as value 100%, dot line). Tubulin was used as a loading control. (C,F) Zymogram analyses of 24 h CM harvested in A375 (C) and A375-M6 (F) after 48 h pre-incubation with CM from sen, senR5 fibroblasts or unconditioned DMEM. MMP-2 and MMP-9 activities were detected by gelatin zymography analysis and appeared as transparent bands (shown on the top). ImageJ was used for quantification. Histograms represent the mean of the gelatinolytic activity of melanoma CM measured in three different experiments \pm SD. Results are reported as a percentage of values obtained after pre-incubation with unconditioned DMEM (assumed as value 100%, dot line). * shows statistical significance ($p < 0.05$) and ** a very statistical significance ($p < 0.01$), compared to incubation with CM from sen fibroblasts. # shows statistical significance ($p < 0.05$) compared to incubation with DMEM.

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Supplementary table 1. Primer sequences used for Real Time PCR experiments

Gene	Forward primer	Reverse Primer
IL-1 α	5'-AGTAGCAACCAACGGGAAGG-3'	5'-TGGTTGGTCTTCATCTTGGG-3'
IL-1 β	5'-AAATACCTGTGGCCTTGGGC-3'	5'-TTTGGGATCTACACTCTCCAGCT-3'
IL-6	5'-GTAGCCGCCCCACACAGA-3'	5'-CATGTCTCCTTTCTCAGGGCTG-3'
IL-8	5'-ATAAAGACATACTCCAAACCTTCCAC-3'	5'-AAGCTTTACAATAATTTCTGTGTTGGC-3'
IL-6 R	5'-CATTGCCATTGTTCTGAGGTTC-3'	5'-AGTAGTCTGTATTGCTGATGTC-3'
MMP-2	5'-CAA CCC AGA TGT GGC CAA CT-3'	5'-GGTCCAGATCAGGTGTGTAGCC-3'
MMP-3	5'-CTCCAACCGTGAGGAAAATC-3'	5'-CATGGAATTTCTCTTCTCATCAA-3'
MMP-9	5'-GAACCAATCTCACCGACAGG-3'	5'-GCCACCCGAGTGTAACCATA-3'
uPA	5'-CACGCAAGGGGAGATGAA-3'	5'-ACAGCATTGTTGGTGGTACTT-3'
uPAR	5'-GCCCAATCCTGGAGCTTGA-3'	5'-TCCCCTTGACAGCTGTAACACT-3'
VIM	5'-TGTCCAAATCGATGTGGATGTTTC-3'	5'-TTGTACCATTCTTCTGCCTCCTG-3'
TGF β	5'-CCCAGCATCTGCAAAGCTC-3'	5'-GTCAATGTACAGCTGCCGCA-3'
TGF β -R2	5'-CAACCACCAGGGCATCCA-3'	5'-TCGTGGTCCCAGCACTCA-3'
CXCR4	5'-GCCTTATCCTGCCTGGTATTGTC-3'	5'-GCCAAGAAAGCCAGGATGAGGAT-3'
IGF-1R	5'-GCACCATCTTCAAGGGCAATTTG-3'	5'-AGGAAGGACAAGGAGACCAAGG-3'
SDF-1	5'-GTGTCACTGGCGACACGTAG-3'	5'-TCCCATCCCACAGAGAGAAG-3'
N-cad	5'-GGTGGAGGAGAAGAAGACCAG-3'	5'-GGCATCAGGCTCCACAGT-3'
GAPDH	5'-CAATGACCCCTTCATTGACCTC-3'	5'-AGCATCGCCCCACTTGATT-3'

Notes: IL = interleukin; MMP = matrix metallo-proteinase; uPA = urokinase plasminogen activator; uPAR = urokinase plasminogen activator receptor; VIM = vimentin; TGF β = transforming growth factor beta; TGF β -R2 = transforming growth factor beta receptor 2; CXCR4 = C-X-C chemokine receptor type 4; IGF-1R = insulin-like growth factor 1; SDF-1 = stromal cell-derived factor 1; N-cad = N-cadherin; GAPDH = glyceraldehyde 3-phosphate dehydrogenase



Supplementary Figure 1. Representative photographs of the invasive activity of A375 and A375-M6 melanoma cells. Conditioned media were collected from young fibroblasts (CM young) and from senescent cultures, either untreated (CM sen) or treated (CM senR5) for 5 weeks with 5 μ M resveratrol and were used in invasion experiments. Matrigel chemoinvasion. Melanoma cells were placed in the upper well while unconditioned DMEM, CM young, CM sen and CM senR5 were placed in the lower one. Spontaneous invasion. Melanoma cells were incubated for 48 h with unconditioned DMEM, CM young, CM sen and CM senR5 then were suspended in fresh DMEM, that was also placed in the lower well. Photographs show representative fields (10x) of cells that after 6 hours at 37°C had passed the Matrigel-coated filters, after fixing and staining by the Diff-Quick method.

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