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# Oleuropein aglycone attenuates the pro-angiogenic phenotype of senescent fibroblasts: A functional study in endothelial cells☆

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ABSTRACT

The "senescence-associated secretory phenotype" (SASP) supports a pro-tumoral and pro-angiogenic microenvironment through increased secretion of inflammatory and growth factors. We investigated oleuropein aglycone (OLE, 10 μM, 5 weeks) effect on capillary morphogenesis induced by MRC5 fibroblast SASP in mature (hMVEC) and progenitor (ECFC) endothelial cells. In senescent fibroblasts OLE reduced NFkB signaling and the expression of several SASP factors. The levels of IL-8 and VEGF were decreased in senescent fibroblast-conditioned medium collected after OLE treatment (CM senOLE) compared to that from untreated fibroblasts (CM sen). Pre-incubation with CMsen enhanced invasive activity and capillary morphogenesis in endothelial cells. This angiogenic phenotype was significantly attenuated upon pre-incubation with CM senOLE, along with reduced MMP-2, MMP-9 and uPA secretion by endothelial cells. In conclusion, OLE can modulate angiogenesis indirectly acting on local senescent fibroblasts, a new mechanism possibly contributing to the beneficial effects of the Mediterranean diet against cancer and cardiovascular diseases.

## 1. Introduction

Cell senescence, one of the hallmarks of aging, is characterized by replicative arrest, cell dysfunction and altered gene expression profile (Campisi, 2013). In recent years, much attention has focused on another feature of the senescent cell, i.e. the increased secretion of inflammatory and growth factors, known as the "senescence-associated-secretory-phenotype" (SASP), found to play a role in many age-related diseases, and to cooperate in inducing a tumor-favoring cell microenvironment (Coppé et al., 2008). In the tumor microenvironment, senescent fibroblasts show features of cancer-associated fibroblasts (Campisi, 2013; Davalos, Coppe, Campisi, & Desprez, 2010), and promote proliferation and invasion of cancer cells with several mechanisms (Xing, Saidou, & Watabe, 2010), including the expression of SASP factors (Menicacci, Laurenzana, & et al., 2017). Beside pro-inflammatory and proliferative signaling acting directly on tumor cells, cancer progression also depends on local angiogenesis to support cancer cell growth and dissemination. Pro-angiogenic factors such as IL-8 and VEGF are also released by the SASP (Coppé et al., 2008). In particular, IL-8 activates and induces migration of endothelial cells (Li, Dubey, Varney, Dave, & Singh, 2003; Vegran, Boidot, Michiels, Sonveaux, & Feron, 2011) and has been shown to participate in the promotion of an aggressive phenotype in breast cancer cells in a senescent microenvironment (Ortiz-Montero, Londoño-Vallejo, & Vernot, 2017. Tumor vasculogenesis is an endothelium-dependent process brought about by endothelial progenitor cells (endothelial colony forming cells, ECFCs)

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recruited from the circulation (Laurenzana et al., 2015), similarly to what happens during the embryonal development. In addition, tumor vascularization resembles the post-natal angiogenesis process, where new blood vessels sprouting from existing microvasculature can arise from the proliferation of resident mature microvascular endothelial cells (hMVECs). The endothelium-dependent vessels, deriving from both vasculogenesis and angiogenesis, will progressively become the principal route of blood supply to malignant cells (Liu et al., 2012).

It has been shown in intraperitoneal cancer metastasis, a type of tumor depending heavily on angiogenesis, that senescent human peritoneal mesothelial cells, which accumulate in the peritoneum *in vivo*, can stimulate the proliferation of endothelial cells through a paracrine action (Ksiazek, Jörres, & Witowski, 2012). In line with this finding, we have shown previously that senescent fibroblast SASP creates a vasculogenic environment promoting vessel formation by endothelial progenitor cells (Menicacci et al., 2018). Thus, the possibility to control tumor angiogenesis by acting on senescent cells in the tumor microenvironment to modulate the SASP can provide additional targets in cancer prevention and chemotherapy.

Different natural compounds with anti-aging properties *in vivo* and *in vitro* have shown the ability to modulate the SASP. For example, we have previously shown that the natural stilbene resveratrol has the ability to reduce both the pro-tumoral and pro-angiogenetic effects of SASP in senescent cultured human MRC5 fibroblasts, a well-known model of cellular senescence (Menicacci et al., 2018; Menicacci, Laurenzana, & et al., 2017).

*Olea europaea* phenols, contained in drupes and leaves, are another group of natural antioxidant and anti-inflammatory molecules whose beneficial properties have been repeatedly reported in healthy subjects (Oliveras-López, Berná, Jurado-Ruiz, López-García de la Serrana, & Martín, 2014) and in age-related diseases, including cancer (Parkinson & Cicerale, 2016; Rigacci & Stefani, 2016). Many of these protective effects are attributed to phenolic compounds, among which Oleuropein is one of the most extensively studied. Studies in rodent models of normal (Pitozzi et al., 2012) and accelerated aging (Bayram et al., 2012) have shown improvement in age-related dysfunctions upon administration of olive oil phenolic compounds, which have been proposed as candidates to counteract age-associated diseases such as neurodegenerative (Casamenti & Stefani, 2017; Rigacci, 2015) and cardiovascular disorders (Souza, Marcadenti, & Portal, 2017). Mechanistic studies indicate that these compounds are able to act at different sites, interfering with protein function and gene expression modulation to modify cellular pathways relevant to the aging process (Menendez et al., 2013; Piroddi et al., 2017). In particular, oleuropein aglycone (OLE) has been shown to modulate autophagy (Miceli et al., 2018; Rigacci et al., 2015) and SIRT-1 (Luccarini et al., 2016). We reported previously that a long-term treatment with OLE can reduce senescence markers such as β-galactosidase-positive cell number and p16 protein expression in MRC5 fibroblasts. In addition, inflammation markers such as COX-2 and α-smooth-actin levels, and the secretion of IL-6 and metalloproteases (MMPs) were reduced by the treatment (Menicacci, Cipriani, Margheri, Mocali, & Giovannelli, 2017).

In the present work, we evaluated the ability of OLE to reduce the vasculogenic activity of MRC5 fibroblast SASP. We show here that OLE-treated senescent fibroblasts have a reduced ability to promote new vessel formation *in vitro* by both ECFCs and hMVECs, compared to untreated senescent cells, and that this feature can be mainly ascribed to the reduction of secreted IL-8.

## 2. Materials and methods

### 2.1. Oleuropein deglycosylation

Oleuropein deglycosylation by β-glucosidase was performed as follows: a 10 mM solution of oleuropein in 310 μL of 0.1 M sodium phosphate buffer, pH 7.0, was incubated with 8.90 IU of almond β-glycosidase overnight at room temperature. The reaction mixture was centrifuged at 36,580 *g* for 10 min to precipitate OLE, which was then dissolved in dimethyl sulfoxide (DMSO) with vortexing and sonication. Complete oleuropein deglycosylation was confirmed by assaying the glucose released in the supernatant with the Glucose (HK) Assay Kit (Sigma).

The mass spectra of oleuropein and of the pellet sample dissolved in DMSO, obtained in ESI and negative ionization mode, by a direct infusion in a triple quadrupole (TSQ Quantum Thermo Finnigan), confirmed the substantial total recovery of OLE in the precipitate. A 50 mM OLE stock solution in DMSO was stored at −20 °C and diluted immediately before use.

### 2.2. Cell cultures and treatments

MRC5 cell line are normal human fetal lung fibroblasts purchased from the National Institute of Aging Cell Repository (Coriell Institute, USA). MRC5 were cultured in high-glucose (4500 mg/L) Dulbecco's Modified Eagle's Medium (DMEM, Euroclone) supplemented with 10% foetal bovine serum (FBS, Sigma–Aldrich), 2 mM L-glutamine, 100 units/mL penicillin and 100 μg/mL streptomycin (Sigma–Aldrich) at 37 °C in 5% $CO_2$ humidified atmosphere, and propagated at confluence by trypsinization.

The experiments with MRC5 fibroblasts were conducted on pre-senescent cultures, defined as having undergone more than 45 PDL (Population Doubling Level, calculated according to Menicacci, Cipriani, & et al., 2017), as described previously (Pitozzi et al., 2013). After 5 weeks propagation in culture with or without 10 μM OLE, a non-cytotoxic concentration, these cells became senescent, as described previously (Menicacci, Cipriani, & et al., 2017). Senescent control and oleuropein-treated MRC5 cultures were referred to as sen and senOLE, respectively. Low PDL (< 30) MRC5 fibroblasts were also propagated in culture and referred to as young. Endothelial Colony Forming Cells (ECFCs) were isolated from > 50 mL human umbilical cord blood of healthy newborns, as described previously (Margheri et al., 2011) after maternal informed consent and in compliance with the Italian legislation, and analyzed for the expression of surface antigens (CD45, CD34, CD31, CD105, ULEX, vWF, KDR, uPAR) by flow-cytometry (Margheri et al., 2011). ECFCs were selected as $CD45^-$, $CD34^+$, $CD31^+$, $CD105^+$, $ULEX^+$, $vWF^+$, $KDR^+$ cells and were grown in EGM-2 culture medium (Lonza) supplemented with 10% FBS, onto gelatin coated dishes. Human Microvascular Endothelial Cells (hMVECs) were purchased from Lonza and were grown in the same conditions of ECFCs. All endothelial cells were used in the range of 7–10 passages in culture.

### 2.3. Preparation of conditioned media

EBM plus 2% FBS was added, after washing with PBS, to MRC5 cell cultures in a ratio of 1 mL/100,000 cells, in all experimental conditions (young, sen and senOLE). Conditioned media (CM) from MRC5 (MRC5 CM) were collected after 24 h incubation from untreated young, sen and senOLE treated fibroblasts, and centrifuged at 1500 rpm for 5 min. These conditioned media were referred to as CM young, CM sen and CM senOLE, respectively. The unconditioned medium was indicated as EBM.

### 2.4. Expression of SASP factor: Real Time PCR on MRC5 cells

Total RNA extraction was performed using TRIzol reagent (Invitrogen). RNA was reverse transcribed with iScript cDNA Synthesis Kit using random primers. mRNA expression of IL-6, IL-8, bFGF, TGF-β, MMP-2, MMP3, uPA, uPAR, CXCR4 and 18S-rRNA gene was assayed by Real Time PCR. The primers (IDT, TemaRicerca) sequences are reported in Table 1.

Quantitative RT PCR was performed with an Applied Biosystem 7500 Fast RT PCR System (Applied Biosystem) using a SYBR Green-

**Table 1**
Primer sequences used for Real Time PCR.

| Gene | Forward primer | Reverse Primer |
|---|---|---|
| IL-6 | 5′-GTAGCCGCCCCACACAGA-3′ | 5′- CATGTCTCCTTTCTCAGGGCTG-3′ |
| IL-8 | 5′-ATAAAGACATACTCCAAACCTTTCCAC-3′ | 5′-AAGCTTTACAATAATTTCTGTGTTGGC-3′ |
| bFGF | 5′-CTTTGGCTGCTACTTGGAGG-3′ | 5′-GAAGCTTTCCAGCAAAGTGG-3′ |
| TGFβ | 5′-CCCAGCATCTGCAAAGCTC-3′ | 5′-GTCAATGTACAGCTGCCGCA-3′ |
| MMP-2 | 5′-CAA CCC AGA TGT GGC CAA CT-3′ | 5′-GGTCCAGATCAGGTGTGTAGCC-3′ |
| MMP-3 | 5′- CTCCAACCGTGAGGAAAATC -3′ | 5′- CATGGAATTTCTCTTCTCATCAAA -3′ |
| uPA | 5′-CACGCAAGGGGAGATGAA-3′ | 5′-ACAGCATTTTGGTGGTGACTT-3′ |
| uPAR | 5′-GCCCAATCCTGGAGCTTGA-3′ | 5′-TCCCCTTGCAGCTGTAACACT-3′ |
| CXCR4 | 5′-GCCTTATCCTGCCTGGTATTGTC-3′ | 5′-GCGAAGAAAGCCAGGATGAGGAT-3′ |
| 18S-rRNA | 5′-CCAGTAAGTGCGGGTCATAAG-3′ | 5′-GCCTCACATAACCATCCAATC-3′ |

based detection with the default PCR setting: 40 cycles of 95 °C for 15 s and of 60 °C for 60 s. The "Delta-delta method" was used for comparing relative gene expression results using 18 s as the housekeeping gene.

### 2.5. IL-8 and VEGF detection in MRC5 conditioned media

IL-8 and VEGF were detected in CMs normalized for cell number (see 2.3 paragraph) from young fibroblast cultures, CM sen, and CM senOLE using the Mini TMB ELISA Development Kit (Peprotech, DBA) and RayBio ELISA Kit respectively, according to the manufacturer's instructions. Each sample was tested in duplicate and three independent experiments were performed.

### 2.6. Western blot analyses

Cell aliquots of senescent control and OLE-treated MRC5 cultures were collected at the end of the respective treatments and lysed in RIPA buffer (25 mM Tris–HCl pH 7.6, 150 mM NaCl, 1% NP-40, 1% sodium deoxycholate, 0.1% sodium dodecyl sulfate, SDS) with 1% protease inhibitor Cocktail (Sigma-Aldrich) and disrupted by sonication (Microson XL-2000, Misonix). Lysates were clarified by centrifugation and supernatants collected and stored at −20 °C. Protein content was measured with Bradford assay. Protein aliquots (25–40 μg), were separated by 12% SDS-polyacrylamide gel electrophoresis (SDS-PAGE, NuPAGE, Novex; Invitrogen), transferred to nitrocellulose membranes (Millipore), blocked in 5% skim milk and incubated overnight with the specific primary antibodies: mouse anti-tubulin (Sigma-Aldrich), mouse anti-NFkB (Santa Cruz Biotech.), mouse anti-uPAR (Santa Cruz Biotech.) recognizing the full-length uPAR, CXCR4 (Millipore), TGFβ-R2 (Santa Cruz Biotech.), followed by the suitable HRP-coniugated secondary antibodies (Sigma–Aldrich). All the resulting immunocomplexes were visualized with an enhanced chemiluminescence ECL detection system (GE Healthcare) and quantified by ImageJ software (NIH).

### 2.7. NF-kB immunofluorescence

The primary antibody was the same than that used for western blotting, while the secondary was a Cy3–conjugated goat anti-rabbit IgG (Sigma–Aldrich Chemicals). The coverslips with the immune-labelled cells were mounted with an anti-fade mounting medium (Biomeda, Italy) and analyzed under a Bio-Rad MRC 1024 ES confocal laser scanning microscope (Bio-Rad) equipped with a 15-mW Krypton/Argon laser source. The cells were observed with a Nikon Plan Apo X60 oil immersion objective (Nikon Instruments, Italy) at 595 nm. Series of optical sections (X- and Y-steps: 512 × 512 pixels) were then obtained through the depth of the cells, with a thickness of 1 μm at intervals of 0.8 μm (Z-step). A single composite image was obtained by superimposition of 20 optical sections for each sample. Total NFkB fluorescence intensity and Mander's coefficient (M1), used to assess NF-κB p65 colocalization with the nucleus (DAPI), were determined by ImageJ software.

### 2.8. ECFC and hMVEC treatment with MRC5 CMs

ECFCs or hMVECs were seeded in six-well plates ($12 \times 10^4$/well); once grown to approximately 80% confluency, cells were incubated overnight with 1 mL unconditioned EBM, or different MRC5 CMs, and subsequently recovered by trypsinization for invasion and capillary morphogenesis assays.

### 2.9. Invasion assays in Boyden chambers

Spontaneous invasion experiments were performed in Boyden chambers, with wells separated by 8 μm-pore size polycarbonate filters coated with Matrigel (50 μg/filter). ECFC and hMVEC cultures were pre-incubated overnight with EBM or MRC5 CMs. Then, cells from each incubation group were detached, counted and then re-suspended in 200 μL of the same conditioned media, placed in the upper well of Boyden chambers and incubated overnight. Fresh EBM plus 2% FBS was placed in the lower well. Invasion was performed for 6 h at 37 °C in 5% $CO_2$, then filters were recovered and fixed in methanol. Non-invading cells on the upper surface of the filter were removed with a cotton swab while invasive cells adherent on the lower filter surface were stained and counted using a light microscope. Results were reported as the percentage of cells migrated after pre-incubation with MRC5 CMs compared to those migrated after pre-incubation with EBM.

### 2.10. Capillary morphogenesis

*In vitro* capillary morphogenesis was performed as described (Margheri et al., 2010, 2011) in tissue culture wells coated with Matrigel (BD Biosciences). Following overnight pre-treatment with EBM or MRC5 CMs, ECFCs and hMVECs were re-suspended ($18 \times 10^3$/well in 96 well plates) for 6 h in the same incubation media at 37 °C-5% $CO_2$. Results were quantified at the end of experiment with the Angiogenesis Analyzer tool of Image J software, measuring the number of junctions, branches and tubules. Six to nine photographic fields from three plates were analyzed for each point.

### 2.11. MMPs and uPA zymography analyses in ECFC and hMVEC conditioned media

Following incubation with the different MRC5 CMs, both ECFC and hMVEC cultures were further incubated overnight in control EBM plus 2% FBS medium (1 mL/100,000 cells), and the obtained endothelial cell-conditioned media were collected for zymography analyses. These conditioned media (10–20 μL aliquots) were diluted with 4x Tris–Glycine SDS Native Sample Buffer (Invitrogen, Italy) and loaded onto 10% Novex Zymogram Gelatin or Casein Gels (Invitrogen). After electrophoretic separation, gels were developed following the manufacturer's instructions. After incubation with SimplyBlue Safestain

(Invitrogen) buffer, MMP-2 and MMP-9 gelatinolytic activities were detected as transparent bands in the otherwise homogeneous blue gel and quantified using ImageJ software. uPA caseinolytic activity was detected by casein zymography using a gel overlay assay. Briefly, after electrophoresis, gels were washed twice (2.5% Triton X-100, 100 mL/gel, 30 min each) and carefully overlaid on indicator gels containing agarose (1.14% in Tris-HCl, pH 8.1), milk (15% in Tris-HCl, pH 8.1) and plasminogen (1 mg/ml, 20U), and incubated overnight at 37 °C in humidified atmosphere. After incubation, the casein gel was carefully removed, the indicator gel was stained and uPA activities quantified as transparent bands using ImageJ software.

### 2.12. Statistical analysis

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

## 3. Results

### 3.1. Effect of chronic oleuropein treatment on MRC5 fibroblast SASP

Pre-senescent MRC5 fibroblast cultures (PDL > 45) were treated for 5 weeks, without (sen) and with (senOLE) 10 μM oleuropein concentration, as described in Section 2.2.

Fig. 1A shows the results of Real Time PCR experiments carried out on treated and untreated control senescent fibroblasts, for the expression of SASP-related genes, as follows: interleukins IL-6 and IL-8, chemokine TGFβ and bFGF, proteases MMP-3, MMP-2 and uPA, receptors uPAR and CXCR4. In senOLE treated MRC5 fibroblasts, expression of all reported genes was reduced, with a trend to reduction for IL-6 ($p = 0.9985$), a 20% reduction of TGFβ expression ($p = 0.7585$), and a more pronounced decrease in IL-8, bFGF and CXCR4 (all around 50%), compared to control sen MRC5. Also the expression of MMP-2 was reduced ($p = 0.9646$), with a major effect on MMP-3.

MRC5 CMs were subsequently analyzed for IL-8 and VEGF secretion (Fig. 1B). CM senOLE contained about 50% less IL-8 than control sen CM, where this interleukin was found about five-fold higher that in young MRC5 CM (reported as 100%). We also measured about 30% increase in VEGF secretion by senescent fibroblasts compared to young, and a slight reduction to levels comparable to young in treated senescent cells (CM senOLE, $p = 0.2903$).

Fig. 2A shows the results of western blot experiments carried out on MRC5 cell extracts at the end of OLE treatment. NF-kB content was found about 25% lowered in OLE-treated MRC5 compared to sen untreated fibroblasts. In senOLE fibroblasts the levels of receptor proteins CXCR4, TGFβ receptor 2 (TGFβR2) and uPAR were found reduced to around 70%, 60% and 55% of sen, respectively. Sen and senOLE-treated MRC5 cells were also compared for NF-kB fluorescence intensity and nuclear translocation (Fig. 2B). Sen MRC5 showed an increase in nuclear localization of NF-kB compared to senOLE cells, in parallel with an increment in total NF-kB fluorescence detected by quantitative image analysis.

### 3.2. Effect of MRC5 CMs on the invasive properties of ECFCs and hMVECs

ECFC and hMVEC cultures were incubated overnight with different conditioned media: CM sen, CM senOLE, and unconditioned EBM, respectively, following the experimental design summarized in the graphical abstract. Cells were then collected, counted and used for subsequent analyses herein reported. For invasion experiments (Fig. 3), cells were seeded in the upper chamber of matrigel-coated transwells, with standard EBM plus 2% FBS in all lower chambers (see Section 2.9). After 6 h incubation at 37 °C, cells that had passed through the lower surface of the filters were photographed and counted. Histograms report the percentage of the cell counts, taking the EBM incubation as 100%. A representative set of microphotographs of cells that passed the filter is also shown. It can be seen that hMVECs invasive activity was 30% augmented by the treatment with CM sen, that induced a much stronger increase in ECFCs spontaneous invasion (230%), compared to the incubation with EBM. After pre-treatment with CM senOLE, the number of cells that passed the matrigel traswells was significantly reduced in ECFC cells (from 230 to 170% of control EBM incubation), and it was brought back to basal levels in hMVEC cells.

### 3.3. Effect of MRC5 CMs on tubulogenesis by ECFC and hMVEC

To evaluate the effects of the different CMs on the ability of ECFCs and hMVECs to form new blood vessels, a capillary morphogenesis test on Matrigel was employed, as described in Section 2.10. (Fig. 4). When suspended in CM sen, both cell cultures exhibited a much higher ability to form new tubes and networks compared to those in unconditioned EBM, as shown by an increased number of master junctions, branches, tubules and by tubule and total length (panel B), whereas cells suspended in CM senOLE showed no increase in capillary forming activity.

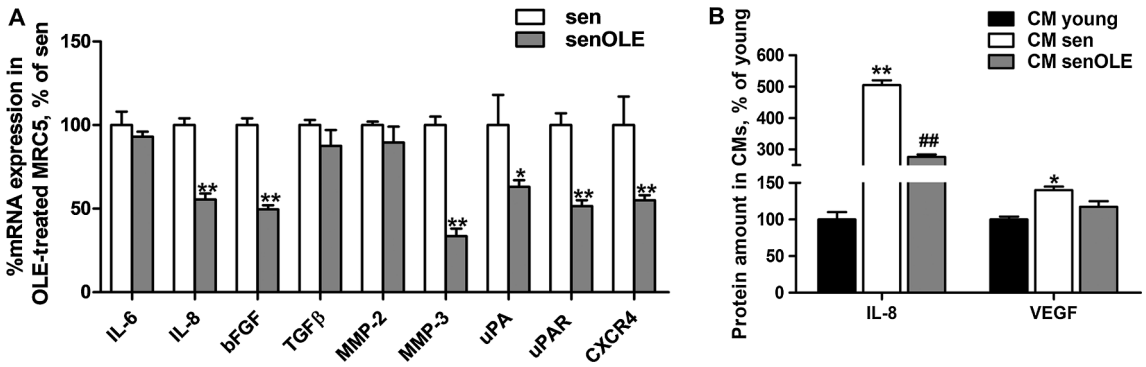


**Fig. 1.** SASP factors expression on MRC5 fibroblasts and IL-8 and VEGF release in conditioned media. MRC5 senescent fibroblasts were treated for 5 weeks with 10 μM oleuropein aglycone (senOLE), along with senescent untreated (sen) cultures. At the end of the treatment, cells were analyzed for the expression of IL-6, IL-8, bFGF, TGF-β, MMP-2, MMP3, uPA, uPAR and CXCR4 by Real Time PCR, using 18S rRNA as housekeeping gene. (A) Histograms report values normalized to sen cultures (assumed as value 100%) according to $2-\Delta\Delta Ct2-\Delta\Delta Ct$ method and represent the mean of three different experiments ± SD. ** shows high statistical significance ($p < 0.01$), compared to sen values. (B) Following the above reported treatment procedure, 24 h-Conditioned Media (CM) were collected from untreated (CM sen), OLE-treated (CM senOLE) and young cultures (CM young). IL-8 and VEGF levels were measured in normalized CM aliquots by an ELISA assay. Results represent the mean of three different experiments ± SD. * shows statistical significance ($p < 0.05$), ** shows high statistical significance ($p < .01$), compared to young values. ## shows high statistical significance ($p < 0.01$), compared to sen values.

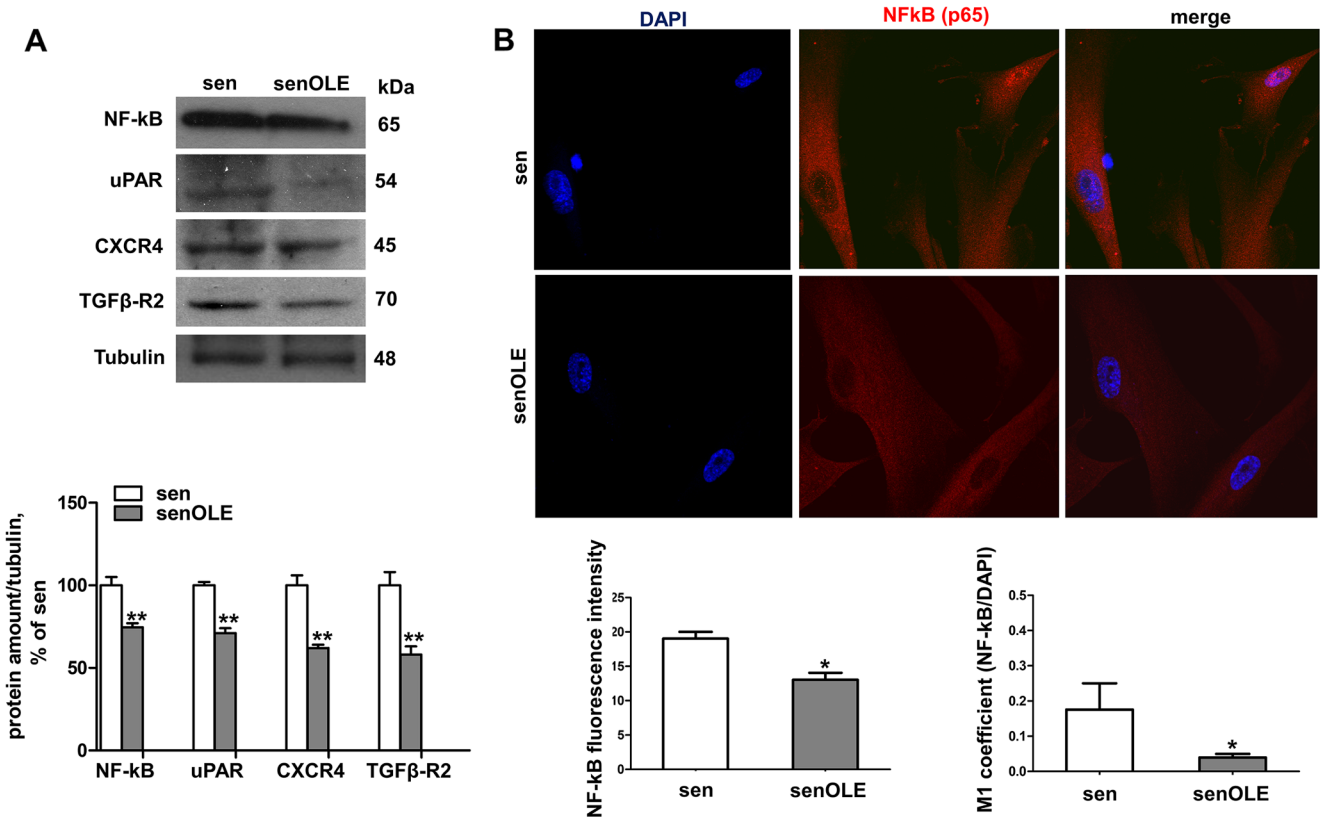


**Fig. 2.** Effect of chronic oleuropein treatment on MRC5 fibroblast SASP. MRC5 senescent fibroblasts were treated for 5 weeks with 10 μM oleuropein aglycone (senOLE), along with senescent untreated (sen) cultures. (A) Western Blotting analysis of NFkB, uPAR, CXCR4 and TGFβR2 in sen and senOLE MRC5. Tubulin was used as loading control. Bands were quantified by Image J. Histograms represent the mean of three different experiments ± SD. The results, reported as percentage, were normalized to sen values (assumed as value 100%). ** shows high statistical significance ($p < 0.01$) compared to sen. (B) NFkB intracellular localization analyzed by immunofluorescence. Histograms show NFkB fluorescence quantification (on the left) and nuclear localization (NFkB/DAPI) by Mander's coefficient (M1, on the right) using Image J software on three different fields for each experimental condition. * shows statistical significance ($p < 0.05$) compared to sen.

### 3.4. Effect of MRC5 CMs on MMPs secretion by ECFC and hMVEC

Zymogram analyses (Fig. 5) of 24 h ECFC-conditioned media revealed that MMP-9 was significantly augmented and MMP-2 was almost unaffected in media conditioned by ECFCs pre-incubated with CM sen, compared to EBM (EBM incubation, reported as 100%). In addition, about 60% and 30% reduction in MMP-9 and MMP-2 active protein secretion, respectively, were found in senOLE-treated, compared to sen cultures.

In hMVEC-conditioned media only MMP-2 was augmented in cells pre-treated with CM sen whereas MMP-9 was unaffected, compared to EBM. Also in these cells, senOLE pre-treatment lowered both MMP-2 (to control levels) and MMP-9 (about 50% reduction) compared to sen.

Finally, in both ECFC and hMVEC the measurement of secreted uPA revealed a strong increase of activity (four and seven-fold, respectively) after incubation with CM sen, compared to EBM. Conversely, CM senOLE pre-treatment reduced uPA activity compared to CM sen only in hMVEC cultures, although to values still much higher than control EBM.

## 4. Discussion

The senescence-associated inflammatory phenotype of MRC5 fibroblast has been repeatedly shown to promote a tumor-favoring and angiogenic microenvironment (Menicacci et al., 2018; Menicacci, Laurenzana, & et al., 2017). The present study shows that OLE attenuates the fibroblast SASP-induced angiogenetic profile of human endothelial cells (hMVECs and ECFCs). In particular, we show that endothelial cells exposed to media conditioned by OLE-treated senescent fibroblasts display reduced cell invasion and capillary-like structures formation activity, compared to untreated senescent fibroblasts. Direct antiangiogenic effects of olive oil or pomace extracts have been shown in endothelial cells, mediated by the down-regulation of genes involved in inflammation, ROS production and angiogenesis (Calabriso et al., 2016; Palmieri et al., 2012). Purified olive phenols such as oleuropein and its metabolite, hydroxytyrosol (HT) and have also been reported to reduce angiogenesis in human vascular endothelial cells through inhibition of the expression and activity of MMP-9 and COX-2 (Scoditti et al., 2012). HT was also reported to inhibit both PGE-2-induced VEGF expression in HT-29 colon cancer cells and endothelial sprouting induced by HT-29 cells in a co-culture system (Terzuoli et al., 2010).

To shed further light on the role of olive phenols in the cell interplay that leads to carcinogenesis, here we report for the first time the activity of OLE in trans-inhibiting the angiogenetic activity of endothelial cells, by acting on another cell type involved in the generation of a permissive tumor environment, i.e. the senescent fibroblast. We have previously shown that OLE reduces SASP in MRC5 senescent fibroblasts (Menicacci, Cipriani, & et al., 2017). In the present work, we assessed the effect of media conditioned by senescent MRC5 cells treated with OLE on the two principal types of mechanism that intervene in vessel development and growth, i.e. vasculogenesis and angiogenesis. Currently, vasculogenesis is considered as occurring in post-natal life, with neo-vessel formation from endothelial progenitor cells (EPCs) ("post-natal vasculogenesis by EPCs"). On the other hand, angiogenesis is the formation of new vessels from an established microvasculature ("sprouting angiogenesis by resident EC") and is therefore responsible for vascular network expansion and remodeling (Laurenzana et al., 2015). ECFCs (the EPC sub-population used in this study) can

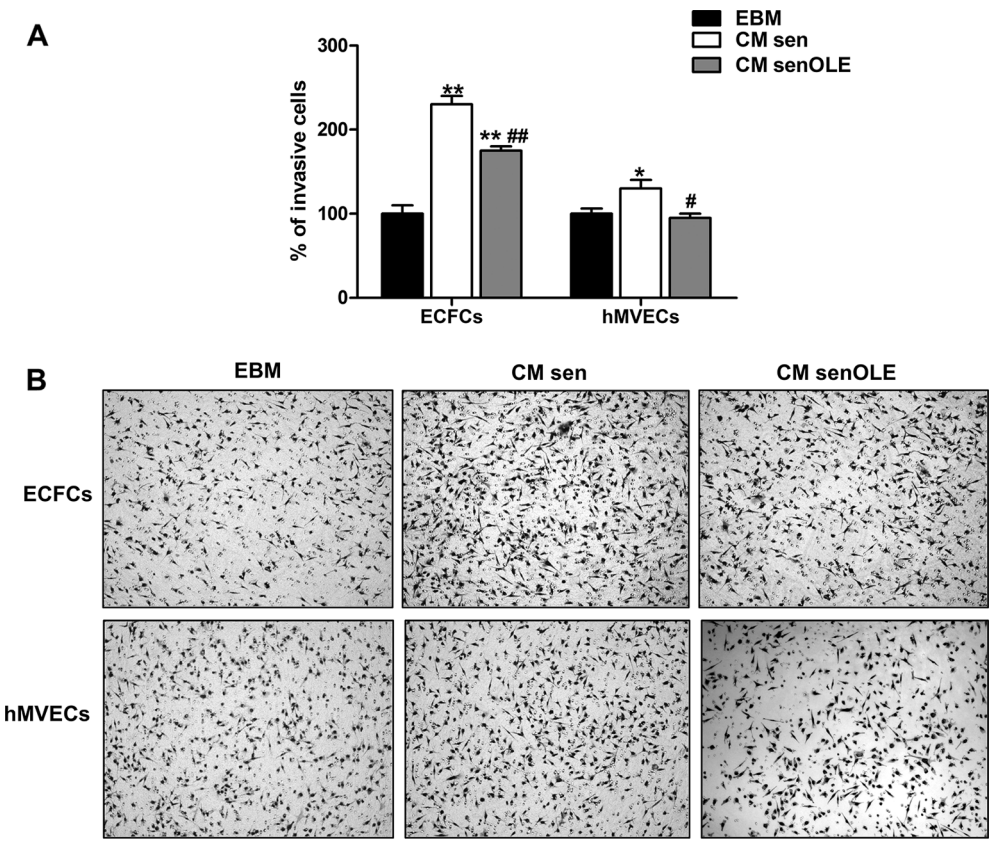


**Fig. 3.** Effect of CM from MRC5 fibroblasts on the invasive activities of ECFCs and hMVECs. ECFCs and hMVECs were incubated overnight with CM sen, CM senOLE or unconditioned EBM (used as a reference condition). Then $2{,}5 \times 10^4$ cells were suspended in the same media of the overnight incubation and placed in the upper well. Fresh EBM was placed in the lower well. (A) Histograms represent the mean of three different experiments ± SD, and results are reported as the percentage of migrated cells after pre-incubation with MRC5 CMs compared to those migrated in control condition (assumed as value 100%). * shows statistical significance ($p < 0.05$), ** shows high statistical significance ($p < 0.01$) compared to unconditioned EBM. # shows statistical significance ($p < 0.05$), ## shows high statistical significance ($p < 0.01$), compared to CM sen. (B) Representative photographs (10×) of migrated cells.

contribute to sprouting angiogenesis not only for their incorporation into new vessel lumen but also secreting numerous pro-angiogenic factors able to enhance proliferation, survival and function of mature ECs (Laurenzana et al., 2015). During angiogenesis, local proteolysis at the basement membrane of the parent vessels is a necessary step for endothelial cell migration.

Many studies, including gene deletions in mice, have shown the critical role of matrix metalloproteases (MMPs) and of the urokinase-type plasminogen activator receptor (uPAR)/associated plasminogen activator (uPA)/plasmin system in the initiation of angiogenesis (Breuss & Uhrin, 2012; Koziol, Martín-Alonso, Clemente, Gonzalo, & Arroyo, 2012; Pepper, 2001; Senger & Davis, 2011; Van Hinsbergh, Engelse, & Quax, 2006). MMP-2, MMP-9 (referred to as gelatinases) and MT1-MMP (membrane type-1 MMP) stimulate angiogenesis not only by ECM degradation, but also by activation of ECM-trapped growth factors and cytokines, recruitment of EPCs and degradation of inhibitors (Pepper,

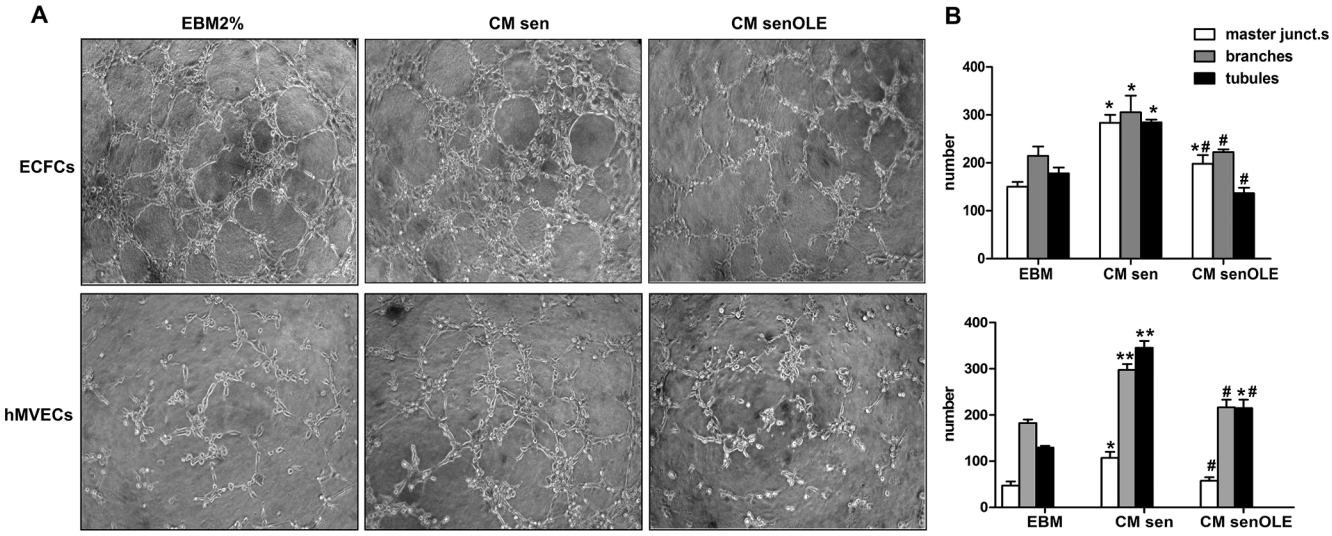


**Fig. 4.** Effect of CM from MRC5 fibroblasts on capillary morphogenesis of ECFCs and hMVECs. ECFCs and hMVECs were incubated overnight with CM sen, CM senOLE and EBM (reference medium). Then, cells were suspended in the same media of the overnight incubation and capillary morphogenensis assays were performed for 6 h at 37 °C. (A) Representative photographs (10×) of capillary-like structures are shown after incubation with CM sen, CM senOLE and EBM. (B) Quantification of capillary network of ECFCs by the Angiogenesis Analyzer Image J tool. Histograms represent the mean ± SD of the number of master junctions, branches and tubules respectively. Data are representative of measures obtained from 6 to 9 fields. * shows statistical significance ($p < 0.05$), ** shows high statistical significance ($p < 0.01$) compared to unconditioned EBM. # shows statistical significance ($p < 0.05$), compared to CM sen.

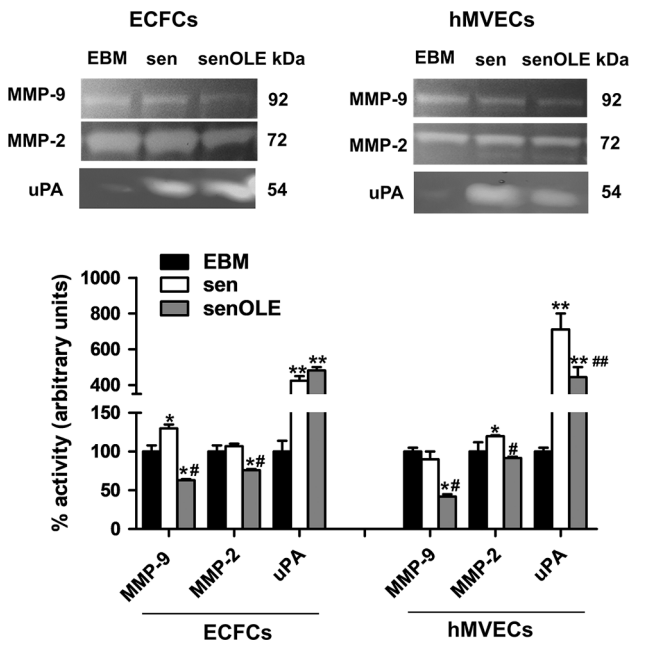


**Fig. 5.** Effect of CM from MRC5 fibroblasts on MMPs and uPA secretion of ECFCs and hMVECs. Gelatin and casein zymography of 24 h-conditioned media collected from ECFCs and hMVECs previously incubated overnight with CM sen, CM senOLE and EBM (reference medium). Histograms show the quantification of gelatinolytic (MMP-2 and MMP-9) and caseinolytic (uPA) activity, respectively, expressed as mean of three different experiments ± SD. Results, reported as percentage, were normalized to EBM values (assumed as value 100%). * shows statistical significance ($p < 0.05$), ** shows high statistical significance ($p < 0.01$) compared to unconditioned EBM. # shows statistical significance ($p < 0.05$), ## shows high statistical significance ($p < 0.01$), compared to CM sen.

2001; Van Hinsbergh et al., 2006). In addition to their classical function in ECM degradation by their collagenolytic activity, MMP-2 and MMP-9 play a significant role in ECFC differentiation (Wu et al., 2010) and in initiating angiogenesis (Bergers et al., 2000; Huang et al., 2009) respectively. Actually, experiments on capillary tube formation performed in a collagen-rich matrix indicated that MT1-MMP promotes the formation of invading EC tubules, whereas MMP-2 and MMP-9 bound to their cognate cell-surface receptors β3-integrin and CD44, do not, although their presence is strictly required for an efficient angiogenesis (Chun et al., 2004; Senger & Davis, 2011).

The presence of fibrin as the main component of the angiogenesis provisional matrix requires the activity of the uPAR-dependent plasminogen activation system in order to function properly. In fact, although several functional links between MMPs and the fibrinolytic system may affect peri-cellular fibrin degradation, the main cell-associated fibrinolytic system hinges on uPAR and on the uPAR/uPA-triggered plasminogen activation to plasmin (Lijnen, 2002).

Here we show an indirect anti-angiogenic effect of OLE on ECFCs and hMVECs, mediated by the modulation of MRC5 SASP. Our findings confirm that long-term OLE treatment of MRC5 senescent fibroblasts induced a reduction of SASP-related factors, and of the amount and nuclear localization of NF-kB, as we have previously reported in dermal fibroblasts (Menicacci, Cipriani, & et al., 2017). As expected, we found a relevant modulation in SASP-related cytokines and membrane receptors involved in angiogenesis regulation and in the pro-tumoral features of senescent cells (Campisi, 2013). In this regard, we measured in senescent fibroblasts a very significant reduction of uPAR, CXCR4 and TGFbeta R2 following OLE treatment, suggesting a consequent lowering of signaling-mediated fibroblast activation by environmental factors. Similar results were obtained after resveratrol treatment of sen MRC5 fibroblasts (Menicacci, Laurenzana, & et al., 2017). In particular, we observed a decrease in IL-8 gene expression in OLE-treated fibroblasts and a consequent lower level of this cytokine in fibroblast-conditioned media, compared to untreated controls. Functional experiments with ECFCs and hMVECs endothelial cells exposed to media conditioned by senescent fibroblast (CM sen) showed increased invasive properties and capillary morphogenesis in comparison with unconditioned medium. All these features were reduced when ECFCs and hMVECs were exposed to OLE-treated senescent MRC5 conditioned media (CM senOLE). It is important to underline that in these experiments ECFCs and hMVECs were treated with fibroblast-conditioned media that did not contain oleuropein (see Methods), so that the observed effects cannot be attributed to the direct action of OLE.

We then analyzed endothelial cell-conditioned media for the proteolytic activity of uPA, MMP-2 and MMP-9. Zymogram analyses of CM collected from ECFCs and hMVECs, pre-incubated overnight with CM sen, revealed an increase of MMP-2, MMP-9 and uPA activities. Conversely, the exposure of ECFCs and hMVECs to media conditioned by OLE-treated senescent fibroblast resulted in a significant reduction in MMP-9 and MMP-2 active protein secretion, while uPA activity was lowered only in hMVEC cultures. These data suggest that the mechanism underlying the anti-angiogenic effect of media conditioned by OLE-treated fibroblasts is the modulation of the proteolytic activity of MMP-2, MMP-9 and uPA in endothelial cells. It has been shown that the treatment with IL-8 up-regulates MMP-2 and MMP-9 mRNA expression and activity in endothelial cells, enhancing survival and angiogenetic properties (Li et al., 2003). Here we show that OLE is effective in modulating SASP-mediated IL-8 secretion. Therefore, IL-8 reduction in tissue microenvironment and the consequently decreased enzymatic activity of MMP-2 and MMP-9 in endothelial cells represent one of the main mechanisms underlying the ability of OLE to modulate angiogenesis by acting on local senescent fibroblasts.

OLE is a major polyphenol in olive oil which is absorbed in the small intestine (Vissers, Zock, Roodenburg, Leenen, & Katan, 2002) and rapidly detected in plasma at different concentrations depending on dose, formulation and gender (de Bock et al., 2013). The 10 μM (3.8 μg/ml) concentration used in the present work has proved effective in a previous study in senescent dermal fibroblasts (Menicacci, Cipriani, & et al., 2017) and is lower than that previously used in other *in vitro* systems, including cardiomyocytes (Miceli et al., 2018), neuroblastoma cells (Luccarini et al., 2016), and cytotoxicity studies of amyloid fibrils (Leri et al., 2018). Instead, most of the *in vitro* work on the direct effects of oleuropein on angiogenesis has been done with the glycoside form (Palmieri et al., 2012; Scoditti et al., 2012). In clinical settings, most agents are typically given by repeated administration, so it is reasonable to suppose that repeated dosing of OLE may lead to accumulation (Liston & Davis, 2017), thus reaching concentrations in the range of those used *in vitro*, as already reported (Ruzzolini et al., 2018).

In conclusion, our study provides a first evidence that OLE inhibits fibroblast SASP-dependent migration and tube formation of endothelial cells, by the modulation of secreted pro-angiogenic factors in cell microenvironment. These results provide a new mechanistic explanation for the anti-angiogenic activities of olive oil polyphenols, and could potentially contribute to explain the beneficial effect of the Mediterranean diet against cancer and cardiovascular disease.

## Conflict of interest

The authors declare that they have no conflict of interest to report.

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## Ethic Statements

Our research did not include any human subjects and animal experiments.

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