

Activation of mutated TRPA1 ion channel by resveratrol in human prostate cancer associated fibroblasts (CAF)

Eric Vancauwenberghe¹, [Lucile Noyer](#)¹, Sandra Derouiche¹, Loïc Lemonnier¹, Pierre Gosset², Laura R. Sadofsky⁴ Pascal Mariot¹, Marine Warnier¹, [Alexandre Bokhobza](#)¹, Christian Slomianny¹, Brigitte Mauroy^{1,3}, Jean-Louis Bonnal^{1,3}, Etienne Dewailly¹, Philippe Delcourt¹, Laurent Allart¹, Natalia Prevarskaya¹ and Morad Roudbaraki^{1*}.

Affiliations

¹Univ. Lille, Inserm, U1003 - PHYCEL - Physiologie Cellulaire, F-59000 Lille, Equipe labellisée par la Ligue Nationale contre le cancer, Villeneuve d'Ascq, France; Laboratory of Excellence, Ion Channels Science and Therapeutics; Université Lille I Sciences et Technologies, Villeneuve d'Ascq, France

²Laboratoire d'Anatomie et de Cytologie Pathologique du groupement hospitalier de l'Institut Catholique de Lille, Faculté Libre de Médecine, Lille

³Service d'Urologie de l'hôpital St-Philibert

⁴Cardiovascular and Respiratory Studies, The University of Hull, Castle Hill Hospital, Cottingham, Hull HU16 5JQ, UK

Running title: Resveratrol activates CAF TRPA1

This is the peer reviewed version of the following article: Vancauwenberghe E, Noyer L, Derouiche S, et al. Activation of mutated TRPA1 ion channel by resveratrol in human prostate cancer associated fibroblasts (CAF). *Mol Carcinog.* 2017; 56:1851–1867., which has been published in final form at <https://doi.org/10.1002/mc.22642>. This article may be used for non-commercial purposes in accordance With Wiley Terms and Conditions for self-archiving.

***To whom correspondence should be addressed:**

Laboratoire de Physiologie Cellulaire, INSERM U1003
SN3, Université Lille I Sciences et Technologies, 59655 Villeneuve d'Ascq, France

Fax: (33) 3 20 43 40 66
Phone : (33) 3 20 33 64 23
e-mail: morad.roudbaraki@univ-lille1.fr

Grant supports Acknowledgments

This work was supported by grants from Région Nord Pas-de-Calais, Institut National de la Santé et de la Recherche Médicale (INSERM), the Ministère de l'Éducation Nationale de l'Enseignement Supérieur et de la Recherche, La Ligue Nationale Contre le Cancer. S. Derouiche was supported by the Région Nord Pas-de-Calais and Association pour la Recherche sur les Tumeurs de la Prostate (ARTP). The funders had no role in study design, data collection and analysis, decision to publish, or preparation of the manuscript.

Abbreviations: TRPA1, Transient Receptor Potential Ankyrin 1; PCa, prostate cancer; RES, resveratrol; SNP, single nt polymorphism; VEGF, vascular endothelial growth factor; HGF, hepatocyte growth factor; SOCE, store operated calcium entry; TME, Tumor microenvironment; HC-030031, 2-(1,3-Dimethyl-2,6-dioxo-1,2,3,6-tetrahydro-7*H*-purin-7-yl)-*N*-(4-isopropylphenyl)acetamide; A967079, 4-fluorophenyl-2-methyl-1-pentene-3-one oxime; AITC, allyl-isothiocyanate; CAF, Cancer-associated fibroblasts; HEK, human embryonic kidney.

Key words: TRPA1, Prostate cancer, Tumor microenvironment, Resveratrol, Apoptosis.

ABSTRACT

Previous studies showed the effects of resveratrol (RES) on several cancer cells, including prostate cancer (PCa) cell apoptosis without taking into consideration the impact of the tumor microenvironment (TME). The TME is composed of cancer cells, endothelial cells, blood cells and cancer-associated fibroblasts (CAF), the main source of growth factors. The latter cells might modify in the TME the impact of RES on tumor cells via secreted factors. Recent data clearly show the impact of CAF on cancer cells apoptosis resistance via secreted factors. However, the effects of RES on PCa CAF have not been studied so far. We have investigated here for the first time the effects of RES on the physiology of PCa CAF in the context of TME. Using a prostate cancer CAF cell line and primary cultures of CAF from prostate cancers, we show that RES activates the N-terminal mutated [Transient Receptor Potential Ankyrin 1 \(TRPA1\)](#) channel leading to an increase in intracellular calcium concentration and the expression and secretion of growth factors (HGF and VEGF) without inducing apoptosis in these cells. Interestingly, in the present work, we also show that when the prostate cancer cells were co-cultured with CAF, the RES-induced cancer cell apoptosis was reduced by 40%, an apoptosis reduction canceled in the presence of the TRPA1 channel inhibitors. The present work highlights CAF TRPA1 ion channels as a target for RES and the importance of the channel in the epithelial-stromal crosstalk in the TME leading to resistance to the RES-induced apoptosis.

INTRODUCTION

Prostate cancer (PCa) is one of the major causes of cancer-related death in men in western countries. Androgen-deprivation therapy (ADT) coupled to chemotherapies to prevent androgens from binding to the androgen receptor (AR) has been the norm for prostate cancer treatment over the recent decades. Despite early success in suppressing prostate tumor growth, in most cases the emergence of hormone-refractory cancer cells leads to tumor growth in a hormone-refractory manner associated with an increased risk of metastasis. In these cases of advanced PCa, hormonal manipulations or chemotherapies are essentially palliative with no effective cure for advanced PCa. There is thus an urgent need to investigate the mechanisms of resistance in PCa in order to design new therapeutic drugs.

In recent years, the importance of dietary components such as anti-oxidants in disease prevention has become an important area of research. Resveratrol (RES), a natural polyphenol found in red wine and foods such as grapes and peanuts, has been suggested to have potent chemo-preventive properties and has been reported to inhibit distinct phases of carcinogenesis *in vitro* [1]. Previous studies have demonstrated that resveratrol could not only induce apoptosis directly in PC-3 and LNCaP PCa cells [2,3], but could also sensitize LNCaP and other cell types to apoptosis induced by TRAIL and chemotherapeutic agents [4-6]. The mechanism of sensitization has been reported to involve down-regulation of the expression of survivin, a member of the IAP family [4,5] and altered the expression of Bcl-2 family proteins [4-6]. Thus, apart from a role in chemoprevention, RES has been suggested to have a potential to be used therapeutically to enhance the effects of chemotherapeutic agents by regulating sensitivity to apoptosis.

However, all these studies were only performed on isolated prostate cancer cells without taking into consideration the TME where several cell types are in communication *via* soluble factors. Indeed, carcinomas in general are composed of two interdependent cell components: the neoplastic epithelial cells and the supporting tumour stroma, which plays decisive roles in pivotal processes such as tumour cells proliferation, invasion and tumor vascularization [7-9] *via* the secretion of growth factors and cytokines. Following epithelial changes in carcinogenesis, the surrounding stroma is modified by cancer cell-derived factors. These

modifications drive the emergence of the characteristic reactive stroma: modified stromal cells secreting extracellular matrix proteins and soluble factors (cytokines, growth factors), which in turn play important roles in initiation and/or progression of certain carcinomas, including breast and prostate cancers. The stromal cells are shown to secrete a great number of cytokines and growth factors including the angiogenesis promoting factor vascular endothelial growth factor (VEGF), fibroblast growth factor (FGF) and hepatocyte growth factor (HGF). HGF has been shown to be abundantly expressed and secreted in the tumor microenvironment and is known to promote proliferation and cell motility of epithelial cells expressing the HGF receptor c-Met. The stromal secreted factors such as HGF is also described to be involved in the resistances developed by the patients under treatment by chemotherapeutic agents. In this context, a growing number of studies have demonstrated the interest of investigating the relationship between epithelial tumor cells and their microenvironment in order to improve available chemotherapeutics treatments. Alteration of the secretion of these stromal factor could impact the initiation and/or progression of prostate cancers and the identification of the modulators of these secretions might constitute interesting targets in the PCa chemotherapies and PCa treatments in general. Ion channels are integral membrane proteins that form a pore to allow the passage of specific ions by passive diffusion. A rise in free cytosolic Ca^{2+} concentration ($[\text{Ca}^{2+}]_c$) near the plasma membrane due to Ca^{2+} influx through the membrane Ca^{2+} channels is the main mechanism inducing exocytosis [10-12]. In the context of cancer, alterations in Ca^{2+} homeostasis with modulation of ion channel expression and/or activity could be essential during carcinogenesis or its progression by modulating processes including cell growth, differentiation, migration or secretion.

Previously published data show that environmental factors could modulate ion channel activity or expression [13]. Recently, RES has been shown to modulate calcium signaling in epithelial PCa cells by decreasing Endoplasmic Reticulum (ER) calcium storage and store operated calcium entry (SOCE) [14]. It is conceivable that RES modulates calcium signaling in PCa cancer-associated fibroblasts (CAF) and thereby modifies the secretion and/or expression of growth factors by these cells and subsequently the physiology of the cancer cells in tumor microenvironment. However, the data concerning the impact of RES on calcium signaling in PCa CAF is missing.

Given the importance of the cancer-associated fibroblasts (CAF) of the tumor microenvironment, we studied the impact of RES on PS30 cells, a prostate cancer CAF cell line and on primary cultured CAF derived from human prostate cancer. In the present work,

we show that RES activates a mutated [Transient Receptor Potential Ankyrin 1](#) (TRPA1) calcium channel, an ion channel of the TRP family. [Recently, we showed the expression of TRPA1 channel PCa CAF and its involvement in growth factors secretion by these cells \[15\].](#) The activation of the channel by RES induced an increase in intracellular calcium concentrations and the expression and secretion of growth factors (HGF and VEGF) without inducing apoptosis in these cells. Interestingly, we also show here that when the prostate cancer cells were co-cultured with CAF, the RES-induced apoptosis in epithelial cancer cells was reduced by 40 %, an apoptosis reduction canceled in the presence of the TRPA1 channel inhibitors. These data show the importance of the CAF cells on the effects of RES on prostate cancer cells in the TME. Moreover, the present work highlights CAF TRPA1 channels as a target for RES and the importance of the channel in the epithelial-stromal crosstalk leading to the resistance to RES-induced apoptosis. Our data suggest the targeting of the CAF TRPA1 channel along with the use of RES as an anti-cancer agent to induce the apoptosis of the prostate cancer cells.

EXPERIMENTAL PROCEDURES

Chemicals and antibodies

All chemicals, agonists and growth factors were from Sigma-Aldrich. Allylthiocyanate (AITC), HC-030031, A-967069 and resveratrol were dissolved in DMSO and then diluted to the desired concentration on the day of the experiments. Antibodies were from commercial sources as follows: Rabbit anti-hTRPA1 (Alomone Labs), mouse anti- α -actin and mouse anti-Vimentin (Dako, France), Rabbit anti-Calnexin (Santa Cruz Biotechnology) and horseradish peroxidase-conjugated secondary antibodies (Santa Cruz Biotechnology). Alexa fluor 488-labeled anti-rabbit IgG (Molecular Probes).

Cell lines

LNCaP, PC-3 and DU145 prostate cancer cell lines were obtained from the American Type Culture Collection (ATCC, Manassas, VA, USA) [and cultured as recommended in RPMI 1640 \(Gibco, Life Technologies, France\) supplemented with 10% fetal bovine serum \(Gibco, Life Technologies, France\), and 2 mM L-glutamine \(Gibco, Life Technologies, France\). Cells were routinely grown in 50 ml flasks \(Nunc, PolyLabo\) in a humidified atmosphere at 37 °C \(95% air–5% CO₂\). For calcium imaging and immunofluorescence studies, cells were subcultured on glass coverslips in petri dishes \(Nunc\) using trypsin.](#)

PS30 cell line was derived from a prostate cancer patient. To obtain these cells, primary human prostate stromal explants were infected with an amphotropic retrovirus encoding the E6/E7 open reading frame of the human papillomavirus type 16. The cells were then characterized for the expression of myofibroblasts markers [16].

The cell line was a generous gift of Dr A. Oganessian, Department of Anaesthesiology & Pain Medicine, University of Washington (Seattle, Washington, USA). These cells were cultured in RPMI 1640 or DMEM supplemented with 10% FCS.

Tissue specimens and primary cell cultures

Human PCa biopsies were obtained from consenting patients following local ethical considerations. All experiments involving patient tissues were carried out under approval number 'CP 01/33', issued by the 'Comité Consultatif de Protection des Personnes dans la Recherche Biomédicale de Lille'. For primary culture of Prostate Cancer Stromal Cells (PrCSC) (Cancer-Associated Fibroblasts, CAF), portion of prostate tissue suspected of carcinoma was incised, and one half of the sliced tissue was submitted for immediate microscopic examination on cryostat sections. After establishment of the diagnosis of adenocarcinoma, the remaining half of the tissue was used for primary culture. The tissue was cut into multiple minute cubes, placed on a plastic surface, and grown in Phenol-red-free RPMI 1640 containing charcoal-stripped Fetal Calf Serum (FCS) (CS-RPMI) (Life Technologies, Inc., Gaithersburg, MD). As soon as outgrowths formed around the tissue fragments, cells were trypsinized and cultured for calcium imaging, immunofluorescence, RT-PCR and western blot experiments.

Co-cultures

PS30 and DU145 cells were seeded in 24-well plates at 70% confluence separated by an insert with pores of 0.4 μm diameter pores in order to study paracrine interactions via secreted factors. Cells were incubated in RPMI 1640 supplemented with 10% FCS, pre-treated or not with TRPA1 inhibitor (HC-030031, 50 μM) for 24h and then, treated with or not with RES (1-50 μM , 48h). The cells were then harvested by trypsinization and used for Hoechst staining assays as described below.

RT-PCR analysis of mRNA expression

PS30 cells and primary cultured CAF cells were treated by RES and or TRPA1 inhibitors at the indicated concentrations for 48h and then total RNA were isolated and RT-PCR

experiments were performed as described earlier [17]. The PCR primers and the target sequences for siRNA in this study were designed on the basis of established GenBank sequences and synthesized by Eurogentec (Angers, France) and are detailed in Table 1.

Cell transfections

For siRNA experiments, equal numbers of cells from the same culture were seeded, transfected overnight either with 25 nM of control siRNA (targeting Luciferase mRNA) (Eurogentec, Angers, France), or with 2 different siRNA raised against TRPA1 mRNA (siTRPA1-1, siTRPA1-2). The Hiperfect transfection reagent (Qiagen Inc., Courtaboeuf, France) was used to transfect the cells according to the manufacturer's instructions. The medium was changed 24 h after transfections and cells were incubated for a further 24h before performing experiments. The target sequences for TRPA1 siRNA are given in Table 1. The efficiency of the siRNA was validated by RT-PCR and by Western Blot studies conducted on PS30 cell line and primary cultured CAF (data not shown).

Immunofluorescence studies

The protein expression studies in PCa cells were carried out using either by immunofluorescence staining. Cell medium was removed and cells on coverslips were washed two times with Phosphate Buffered Saline (PBS) before fixing them in 2% paraformaldehyde (PAF) for 15 min at room temperature. After three 5 min washes in PBS, cells were blocked in a solution containing 0.2 % BSA , 0.1% triton X-100 and 3% donkey serum for 30 min at room temperature, then incubated overnight at 4°C with primary antibodies. All antibody stock solutions were made at a concentration of 250 µg/ml. The antibodies and the dilutions used were: TRPA1 (1:200), α-actin (1:100), Vimentin (1:100). After three 5 min washes with PBS, cells were incubated with the corresponding secondary antibody Alexa fluor 488-labeled anti-rabbit or anti-mouse IgG (Molecular Probes, 1:1000) for 1h at room temperature. The cells on coverslips were then incubated in PBS containing 50 ng/ml DAPI for 10 min at room temperature to stain the nuclei. After rinsing twice in PBS, the slides were mounted with Mowiol and then analysed either by confocal microscopy (Zeiss LSM 700; acquisition parameters: objective 40x/1.3 thickness of confocal slide, 1 µm) or by phase contrast microscopy.

Western Blot Assay

Primary cultured CAF cells and PS30 cells were cultured to 80% confluence and total proteins were extracted. Protein samples (20 µg) were subjected to SDS-PAGE on either 6% or 10% acrylamide gels and transferred to a PVDF membrane by semi-dry western blotting (Bio-Rad). The membranes were blocked in TNT buffer (Tris-HCl, pH 7.5, 140 mM NaCl, and 0.05% Tween 20) containing 3% milk for 45 min at room temperature. The membranes were then incubated with primary antibodies overnight at 4°C. All antibody stock solutions were made at a concentration of 250 µg/ml. The antibodies and the dilutions used were: TRPA1 (1:500), α-actin (1:200), Vimentin (1:500) and Calnexin (1:1000).

After three washes of 10 min each in TNT buffer, the membranes were incubated with the corresponding horseradish peroxidase-linked secondary antibodies (Zymed Laboratoires Inc., San Francisco, CA, USA) for 1 h at room temperature. After three 10 min washes in TNT buffer, the membrane were processed for chemiluminescent detection using Supersignal West Dura chemiluminescent substrate (Pierce), according to the manufacturer's instructions. For the comparison of the protein expression levels in different samples, normalization was performed by using the image density analysis of the calnexin proteins bands as an internal standard. Then, the ratios were compared to control sample by setting the ratio value for control cells to 1.

Calcium Imaging

Calcium imaging experiments were performed in Hank's Balanced Salt Solution (HBSS), containing 142 mM NaCl, 5.6 mM KCl, 1 mM MgCl₂, 0–10 mM CaCl₂, 0.34 mM Na₂HPO₄, 0.44 mM KH₂PO₄, 10 mM HEPES and 5.6 mM glucose, using Fura-2/AM as the calcium dye. The osmolarity and pH of external solutions were adjusted to 310 mOsm l⁻¹ and 7.4, respectively. Cytosolic calcium concentration was measured using Fura-2/AM loaded cells. PS30 and HEK293 cells were loaded for 45 min at room temperature with 5 µM Fura-2/AM prepared in HBSS and subsequently washed three times with the same dye-free solution. The coverslip was then transferred into a perfusion chamber on a Olympus IX70 microscope equipped for fluorescence. Fluorescence was alternatively excited at 340 and 380 nm with a monochromator (Polychrome IV, TILL Photonics GmBh, Planegg, Germany) and was captured after filtration through a long-pass filter (510 nm) by a MicroMax 5 MHz CCD camera (Princeton Instruments, Evry, France). Acquisition and analysis were performed with the Metafluor software (Universal Imaging Corp., West Chester, PA, USA). All recordings were carried out at room temperature. The cells were continuously perfused with the HBSS solution and chemicals were added *via* the perfusion system.

To represent the variation in $[Ca^{2+}]_c$, the fluorescence intensity ratio represented by F340/F380 was used as an indicator of changes in cytosolic Ca^{2+} concentration. For calcium imaging experiments using the Fluo-4 calcium probe, cells were loaded with Fluo-4/AM (5 μ M) for 30 min at 37°C. Cells were then washed in HBSS and processed for the effects of RES. The Fluo-4 dye was excited in loaded cells at 470-480nm and the emission was measured at 525 nm, reflecting the intracellular free calcium concentrations. Each experiment was performed on 35–45 cells in triplicate and repeated at least four times on different cell cultures and a representative figure is presented.

Enzyme-linked Immunosorbent Assay (ELISA)

PS30 cells (5×10^4 cells) were incubated in RPMI supplemented with 0.5% FCS, and treated with RES at different concentrations (10, 25 and 50 μ M) with or without TRPA1 inhibitors (HC-030031, 50 μ M; A-967079, 1 μ M) or activator (AITC, 30 μ M) for 24h, in 12-well plates at 80% of confluence. The supernatants of the cell cultures were then collected, centrifuged and processed for HGF and VEGF detections by ELISA Assays using an immunoassay kit (Abcam, Paris, France) according to the manufacturer's instructions.

Cell growth and cell cycle analysis

Cell viability was assessed by a colorimetric MTS/PMS assay (CellTiter 96) according to manufacturer's instructions. For cell cycle analysis, approximately 5×10^5 PS30 cells were fixed in 70% ethanol at -20°C for 30 min. After fixing, cells were pelleted by centrifugation, washed three times with PBS at 4°C , resuspended in 100 ml of PBS, treated with 100 ml of RNase A (1 mg/ml; Ambion) for 15 min, and stained with propidium iodide (Sigma) at a final concentration of 50 $\mu\text{g/ml}$ for 45 min. Propidium iodide fluorescence was measured at 520 nm (excitation at 488 nm) using a flow cytometer (CyAn ADP Analyzer, Beckman Coulter). Data were interpreted using ModFit LT. Each experiment was repeated at least three times.

Apoptosis Assays

Two techniques were used to study the apoptosis in epithelial cells: TUNEL staining and Hoechst 33342 dye staining. TUNEL staining was conducted using the *in situ* Cell Death Detection kit, TMR red, following manufacturer's recommendations [18] on LNCaP cells trypsinized and attached to glass slides by Cytospin and fixed in 70 % methanol at -20°C for 20 min. All the nuclei were counter-stained with DAPI dye (50 $\mu\text{g/ml}$) for 10 min at room

temperature in the dark in order to establish an apoptotic cell nuclei counting (red) on the total population (blue). Then, coverslips were mounted with mowiol and examined under fluorescence microscopy and the apoptotic and non-apoptotic cell nuclei were counted in 10 randomly chosen fields. The percentage of the apoptotic cells in CTL conditions is considered as 100 %.

For Hoechst staining of cells nuclei, treated and untreated cells were harvested by trypsinization and by centrifugation for 5 min, washed with 1 ml PBS and attached to glass coverslips by cytospin then, fixed in 70% methanol at -20°C for 15 min. The cells nuclei were then stained with Hoechst 33342 dye ($4\ \mu\text{g}/\text{mL}$) (blue fluorescent) for 15 min at room temperature in the dark, mounted with mowiol and examined by fluorescence microscopy by counting the apoptotic and non-apoptotic cell nuclei in 10 randomly chosen fields.

Statistical analysis

Plots were produced using Origin 8.0 (Microcal Software, Inc., Northampton, MA). Results are expressed as mean \pm S.E. Statistical analysis was performed using unpaired *t* tests or ANOVA tests followed by either Dunnett (for multiple control *versus* test comparisons) or Student-Newman-Keuls post-tests (for multiple comparisons). Student's *t*-test was used for statistical comparison of the differences and $P < 0.05$ was considered significant.

RESULTS

Effects of resveratrol on prostate cancer associated fibroblast cells

Although resveratrol (RES) is known to induce apoptosis or increase sensitivity to apoptotic stimuli in prostate epithelial cancer cell lines such as LNCaP or PC-3 cells, its effect on prostate CAFs has not been studied so far. In this context, we used an immortalized human prostate cancer stromal cell line, PS30 cells, as well as primary cultured human prostate cancer CAF cells in order to verify if the physiology of these cells could be altered by RES. The cells were treated with different concentrations of RES ($1\text{-}100\ \mu\text{M}$) for 72h, then, the cells were examined under light microscope. As shown in [Figure 1A](#), the RES treatment induced a dose-dependent modification of the CAF cells shape. Indeed, the concentrations of RES below $25\ \mu\text{M}$ failed to induce any changes in cell shape whereas for higher concentrations ([50 \$\mu\text{M}\$](#)), resulted in thinner and more elongated morphology compared to the control cells. As shown in [Figure 1B](#), the morphological modifications were accompanied by a dose-dependent decrease in α -actin and an increase in vimentin expression, the markers for smooth muscle cells and fibroblast respectively. In the same way, MTS/PMS viability assays were also performed on PS30 cells treated with RES at different concentrations ($0,1$ to $100\ \mu\text{M}$) for 72h.

As shown in [Figure 1C](#), for concentrations of 0,1 to 25 μ M, RES failed to affect significantly the number of living cells, but higher concentrations (50 and 100 μ M) induced a decrease of the cell viability for more than 30% compared to control cells. These effects were not accompanied by any morphological signs of cytotoxicity such as cell rounding or floating in the medium, and Hoechst dye-stained nuclei analysis and counting showed that this decrease in living cell number induced by RES was not linked with the cell death by apoptosis or necrosis ([Figure 1D](#)). Moreover, cell cycle study by flow cytometry on PCa CAFs treated with RES (50 and 100 μ M) for 72h exclude apoptosis in these cells following RES treatment and show that proportion of cells in G0/G1 phase increase with RES concentration ([Figure S1](#)). Thus, in contrast to epithelial cancer cells (LNCaP or PC-3), RES failed to induce cell death in prostate CAFs but seemed to elicit morphological changes associated with the modifications of cell markers (α -actin and vimentin) expression.

CAFs are known to promote cancer development and metastasis through paracrine interactions with epithelial cancer cells via cytokines and growth factors secretion. We therefore studied if RES could modulate PS30 cells secretory activities. For this, the secretion rates of two growth factors were evaluated by ELISA assays in the conditioned media (CM) obtained by PS30 cells treated or not with RES for 24h. As shown in [Figures 1E and 1F](#)), RES (10-50 μ M) induced an increase in the secretion of two growth factors actively involved in carcinogenesis: Hepatocyte Growth Factor (HGF) ([Figure 1E](#)) and Vascular Endothelial Growth Factor (VEGF) ([Figure 1F](#)) compared to control. Moreover, intracellular Ca^{2+} chelation with the incubation of the cells with BAPTA/AM decreased the basal secretion of HGF and VEGF by more than 2 fold ([Figures 1E and F](#)) suggesting that the basal secretion is a Ca^{2+} -dependent process in CAF. It is thus possible that RES is able to induce a modulation of the ion channels activity leading to a cytosolic calcium rise and subsequent secretion.

Resveratrol induces Ca^{2+} entry in prostate CAFs

In order to study whether the effects of RES on CAF gene expression and growth factor secretions are mediated by the activation of calcium signaling pathway in these cells, we performed Ca^{2+} imaging experiments. As shown in [Figure 2](#), RES induced a rapid rise in cytosolic free calcium concentrations ($[\text{Ca}^{2+}]_c$) in PS30 cells in a dose-dependent manner ($\text{EC}_{50}=6,49\mu\text{M}$) ([Figure 2B](#)). The RES-induced calcium response consisted in a rapid rise followed by a sustained plateau obtained for the concentrations of $\text{RES}>1\mu\text{M}$. For these experiments, we used Fura-2/AM, a ratiometric fluorescent Ca^{2+} dye, where cytosolic Ca^{2+} levels are determined by measuring the emission of fluorescence at 510 nm following

alternate excitation at 340 nm (F340) and 380 nm (F380) and the F340/F380 ratio reflects the variation of the $[Ca^{2+}]_c$. Previous works suggest that RES might interfere with Fura-2 intracellular Ca^{2+} measurements with a light absorption at 340 nm and emission at 510 nm thus modifying the F340 without affecting F380 values [19,20]. We therefore performed experiments and analysis to verify this possibility in order to validate the effects of RES on $[Ca^{2+}]_c$ in PS30 cells. First, for each calcium imaging experiment using Fura-2, we checked the variations of fluorescence after excitations at 340nm and 380 nm for each calcium imaging experiment performed with Fura-2. In all experiments with RES for the concentrations tested, the F340 and F380 showed an anti-parallel variation (Figures 2C and 2D), suggesting that the calcium response observed with RES treatment was not due to the interferences of the RES with Fura-2 fluorescence. In a second series of experiments, we used Fluo-4, another fluorescent Ca^{2+} dye with an excitation and emission wavelengths of 470 and 525 nm respectively. As shown in Figure 2E, the application of RES on Fluo-4 loaded cells increased the emitted fluorescence at 525nm (F470), suggesting an increase in $[Ca^{2+}]_c$. Taken together, the decrease of F380 values simultaneously with the increase of F340 values for Fura-2 and the increase of F470 values for Fluo-4 after RES application (25 μ M) on PS30 cells confirm that RES induces a Ca^{2+} increase in these cells. Moreover, the absence of response when the cells were incubated in a HBSS medium without Ca^{2+} demonstrates that RES induces a Ca^{2+} entry and not a mobilization from intracellular Ca^{2+} stores (Figure 2F). This implies that RES activates plasma membrane ion channels to induce a Ca^{2+} entry in prostate CAFs.

Resveratrol (RES) activates TRPA1 in prostate CAFs

In order to identify the ion channels involved in the effects of the RES on PS30 cells, different voltage-dependent (nifedipin, flunarizin, Nickel) (Figure S2) and voltage-independent (Capsazepin, SKF96365, BCTC, HC-030031, A-967079) (Figures S2 and S3) calcium-permeable ion channels inhibitors were tested on the RES-induced Ca^{2+} response. Among the inhibitors tested, only TRPA1 inhibitors (HC-030031, A-967079) blocked the RES-induced Ca^{2+} response, suggesting the activation of TRPA1 by RES in human prostate CAF (Figure 3). Indeed, the application of HC-030031 (50 μ M) on RES-induced Ca^{2+} entry suppressed the calcium response (Figure 3A). Similarly, pretreatment of PS30 cells or primary cultured prostate CAF with the TRPA1 inhibitor for 5 min completely inhibited the RES-induced Ca^{2+} entry (Figures 3B and C), the response being restored when the inhibitor was removed from

the medium (Figure 3B). Similar results were obtained by using A-967079 (1 μ M), another specific inhibitor of TRPA1 (data not shown). In the same manner, knockdown of TRPA1 expression by short interference RNA (siRNA) prevented the Ca^{2+} entry induced by RES (Figure 3E). These data show that TRPA1 channel is the main component of RES-induced calcium entry in PCa CAF.

These observations are surprising because RES is known to inhibit mouse and rat TRPA1 channel [21,22]. Using calcium imaging experiments, we therefore studied the effects of the TRPA1 activator Allylthiocyanate (AITC, 30 μ M) on intracellular calcium concentration in the presence or absence of RES (25 μ M) in HEK293 cells transiently transfected with human wild type TRPA1 (wt-TRPA1) expression vector (HEK-wtTRPA1). As presented in Figure 3F, our Ca^{2+} imaging studies showed that AITC (30 μ M)-induced calcium entry was inhibited by RES (25 μ M), whereas in human prostate CAFs, RES activated the channel and failed to affect the Ca^{2+} entry induced by AITC (30 μ M) (Figure 3D).

Functional TRPA1 channels are expressed in prostate CAF cells

After the characterization of TRPA1 ion channel as the main target of RES, we investigated its expression in different human prostate cancer cells and CAF. These studies show that the channel is expressed in PS30 and in primary cultures of prostate CAF (PrCsC) cells, but not in epithelial cancer cell lines (LNCaP, DU145, PC-3) or in primary cultures of epithelial cancer cells (PrCEC) (Figure 4A). The protein expression of TRPA1 channel in PS30 and PrCsC was confirmed by western blot (Figure 4B) and by immunofluorescence studies (Figure 4C). The PS30 and PrCsC were characterized by the expression of the CAF markers vimentin and α -actin as shown for PS30 cells in Figure 4B and 4C. The functionality of TRPA1 channel in PS30 cells was confirmed by Ca^{2+} imaging studies using its activator, AITC. As shown in Figure 4D and 4E, AITC induced a dose-dependent increase in $[\text{Ca}^{2+}]_c$ ($\text{EC}_{50} = 5.19 \mu\text{M}$) in PS30 cells which was suppressed by TRPA1 inhibitors (A-967079, HC-030031) (data not shown). Consistent with the TRPA1 expression studies, the application of either AITC (30 μ M) or RES (25 μ M) failed to induce a calcium response in PCa epithelial cell lines DU145, PC-3 and LNCaP (Figure 4F and 4G). In the latter cell line, at the end of the experiment, the calcium responsiveness of the cells was tested using Thapsigargin (TG, 1 μ M), a SERCA inhibitor known to induce a depletion of the intracellular calcium stores and a subsequent store-operated calcium entry (SOCE) (Figure 4G).

Resveratrol affects epithelia-stromal paracrine interactions in prostate cancer through TRPA1 channel functionality

As mentioned, RES induced a $[Ca^{2+}]_c$ increase by activating TRPA1 channel (Figure 3) and the secretion of growth factors (HGF and VEGF) in human prostate CAF cells (Figure 1E and F). We therefore investigated whether the channel was involved in the secretion of these factors. As shown in Figure 5, TRPA1 inhibition by HC-030031 (50 μ M) prevented the RES-induced secretion of HGF (Figure 5A) and VEGF (Figure 5B). Interestingly, the TRPA1 inhibitor blocked the secretion of the growth factors by at least 50% suggesting the involvement of the channel in the basal secretion of growth factors. As cytoplasmic calcium increase is also known to induce gene expression, we studied whether RES induced growth factors gene expression, and whether TRPA1 channel is implicated.

RT-PCR experiments showed that RES (1 and 10 μ M, 48h) is able to increase HGF and VEGF expression, an effect prevented by TRPA1 inhibition (HC-030031, 50 μ M) (Figure 5C). Taken together, these data suggest that the modulation of TRPA1 channel activity by RES could impact the epithelium-stroma paracrine interactions.

RES is known to induce apoptosis in PCa epithelial cells LNCaP, PC-3 and DU145. We therefore studied whether the impact of RES on epithelial cancer cells is modified when co-cultured with CAF. We first studied the impact of RES on the apoptotic rate of LNCaP cells cultured alone. As shown in Figure 5D, high concentrations of RES (50 and 100 μ M) induced a significant increase of the apoptotic rate of LNCaP cells cultured alone, with rates of 34 and 46% respectively after a 72h incubation. We thus used the concentration of 50 μ M RES in LNCaP/CAF co-culture experiments. We then realized co-cultures of PS30 and LNCaP cells (Figure 5E) separated from each other by an insert (0.4 μ m pore diameter) in order to study the paracrine interactions between these two cells compartments treated with 50 μ M RES in the presence or absence of HC-030031 (50 μ M) for 72h. In these experiments, we observed that the presence of prostate CAFs decreased the apoptotic rate of LNCaP cells in the presence of RES by more than 3.7 fold compared to the control. Interestingly, TRPA1 inhibition by HC-030031 (50 μ M) restored more than 74% of apoptosis normally induced by RES (Figure 5F) in LNCaP cells co-cultured with PS30 cells. These results were confirmed by TUNEL assay realized in the same conditions (Figure 6) showing influence of epithelia-stromal interactions on apoptosis induced by RES and the possibility to restore it by targeting TRPA1 channel expressed by PCa CAFs.

Resveratrol activates mutated TRPA1 in CAF cells

As mouse, rat [20,21] and human (present study, [Figure 3F](#)) wt-TRPA1 channels are inhibited by RES while the channel expressed in PCa CAFs is activated by it, we hypothesized that the TRPA1 expressed in PCa CAFs might present mutations and/or single-nucleotide polymorphisms (SNPs) rendering its activation by RES possible. We thus cloned and sequenced the entire coding sequence of the channel in PS30 cells. For this, using RT-PCR, we cloned separately the N-(1823 bp) and C-terminus (1950 bp) of the coding sequence for TRPA1 expressed in PS30 cells by using the forward and backward primers given in [Table 1](#). The PCR products were then cloned in PGEM-T easy cloning vector (Promega, France) and then sequenced and translated to obtain amino-acids sequences. These sequences were then compared to the wild type TRPA1 amino-acids sequence (NM_007332).

Protein sequences alignment ([Figure 7A](#)) showed 1 mutation (K539R) and 2 SNPs (Y69C and K186N) (according to <http://exac.broadinstitute.org/gene/ENSG00000104321>) in TRPA1 N-terminal domain known to be involved in TRPA1 activity properties [22], while the C-terminal domain amino acids sequences presented 100% identity with wt-hTRPA1 ([Figure 7B](#)). In the light of our results, these mutations/SNP could be involved in the sensitivity of the TRPA1 channel to RES. If this hypothesis is correct, since wt-TRPA1 is inhibited by RES, then the expression of this form of the channel in PS30 cells should induce the formation of heterotetramers of PS30-hTRPA1 and wt-hTRPA1 negatively affecting the sensitivity of the endogenous mutated TRPA1 channels to RES. To verify this, PS30 cells were transfected with either control vector (empty vector) or with the wt-hTRPA1 expression vector. For all transfection studies, the cells were co-transfected with GFP (Green Fluorescent Protein) expression vector in order to select only the transfected cells. Forty-eight hours after transfection, the effects of RES (25 μ M) were investigated in control and wt-hTRPA1 transfected PS30 cells. In these studies, the amplitude of RES-induced Ca^{2+} entry was decreased by 46% (compared to the control cells) in cells transfected with wt-hTRPA1 ([Figure 7C](#)). These data show that the activating effect of RES is reduced on TRPA1 heterotetramers formed by PS30-hTRPA1 and wt-hTRPA1. Taken together, these data suggest that TRPA1 channel activation by RES is specific to the PS30-expressed TRPA1, a channel mutated in its N-terminal domain.

DISCUSSION

Resveratrol (RES), a natural polyphenolic antioxidant, has been reported to possess beneficial effects on pathologies including cancers. RES has indeed been shown to present anti-carcinogenic effects by inducing cell cycle arrest, apoptosis and autophagy-mediated cell death in PCa cells [24-26]. However, these data obtained *in vitro* on isolated epithelial cells did not take in consideration the different cellular components of the tumor microenvironment (TME) and their paracrine interactions. CAFs are important actors known for their secretion of growth factors and cytokines promoting cancer cells transformation and resistance to apoptosis. In this context, prostate CAFs can also interfere with the different cancer treatments, like chemotherapy, by inducing apoptosis resistance in PCa cells. The effects of RES on cancer cells could thus be different in this context where permanent CAF-cancer cell interactions modulate the physiology of both cellular components.

In the present work, we studied the impact of RES on human PCa CAFs for the first time. We showed that RES treatments of PCa CAFs induced a reduction in cell growth at high concentrations ($\geq 50 \mu\text{M}$), but failed to induce apoptosis in these cells. Interestingly, by using primary cultures of CAFs from human PCa and PS30 cell line, we showed that RES also modulates the expression and secretion of HGF and VEGF, two important paracrine growth factors of the TME involved in prostate cancer cell growth, migration, and in tumor angiogenesis. Only a limited number of published works have dealt so far with the impact of RES on fibroblasts physiology. In agreement with our data, RES failed to induce apoptosis in fibroblasts of different origins. Indeed, RES has been reported to be an antioxidant and to induce collagen I gene expression in human gingival fibroblasts without induction of apoptosis [27]. This non-apoptotic effect of RES in fibroblasts has also been also reported by others. For example, Giovannelli *et al* [28] reported protective effects of RES against senescence-associated changes in cultured human fibroblasts. Indeed, these authors showed protective effects against DNA oxidative damage, reduced senescence-associated increases in nuclear size and DNA content, and reduced levels of acetylated H3 and H4 histones and p53 protein in cultured human fibroblasts MRC5. In our studies, we observed that high concentrations of RES induced an inhibition of CAF cells growth with modifications of the α -

actin and vimentin expression suggesting a cell differentiation in PCa CAFs. Consistent with our data, RES has been reported to also reduce both ANG II- and transforming growth factor-induced fibroblast differentiation to the myofibroblast phenotype, indicated by a reduction in α -smooth muscle actin expression and stress fiber organization. This study suggests that RES acts as an anti-fibrotic agent in the myocardium by limiting fibroblasts proliferation and differentiation, two critical steps in the pathogenesis of cardiac fibrosis [29].

In the present work, we observed that RES induced growth factors expression and secretion in PCa CAF (Figures 1E, 1F, 5C). These data suggest that the dietary factor RES modulates in situ CAF-epithelial cells interactions via secreted factors. Moreover, in co-culture experiments, the RES-induced epithelial cells apoptosis was inhibited by at least 40%, clearly showing CAFs protective impact on epithelial cells under RES treatments. In addition, according to previously published data and our results, only the high concentrations (50-100 μ M) of RES induced apoptosis in PCa cells. As the maximal serum concentrations of RES in humans was found to be around 1 to 2 μ M for the highest amounts of RES daily administered [30], the use of the in vitro data obtained on isolated cancer cells is questionable. Indeed, upon oral ingestion, the concentration of intact RES in the circulation is very low [31], due to a rapid conjugation to sulphates and glucuronides, serving as a reservoir of recoverable RES. The final concentration available in the targeted tissue will be determined by the ability of the cells to remove sulphate groups with specific enzymes [32,33] which determine the final concentration available to the targeted tissues. But, using low RES concentrations similar to those that can be found in human serum, we observed an increase in VEGF and HGF mRNA expression (RES 1 μ M) and secretion (RES 10 μ M) suggesting the existence of adverse effects of RES in the context of TME even at low concentrations.

Our data show that the basal and RES-induced secretions were Ca^{2+} -dependent as shown by the use of BAPTA/AM, an intracellular calcium chelator (Figures 1E and F) and the TRPA1 calcium-permeable channel inhibitor HC-030031 (Figures 5A and 5B). These data show that CAFs secretion involves ion channel and/or transporters activation. Ion channels are integral membrane proteins that form a pore to allow the passage of specific ions by passive diffusion. A rise in free cytosolic Ca^{2+} concentration ($[\text{Ca}^{2+}]_c$) near the plasma membrane due to Ca^{2+} influx through the membrane Ca^{2+} channels is the main mechanism inducing exocytosis [10-12]. In cancers, the alterations of Ca^{2+} homeostasis due to the modulation of ion channels expression and activity can be essential during carcinogenesis and/or its progression by modulating processes including cell growth, differentiation, migration or secretion. In this

context, we have shown that TRPA1 was activated by RES (Figures 2 and 3), leading to growth factor expression and secretion.

Transient receptor potential (TRP) ankyrin repeat 1 (TRPA1) is a Ca^{2+} -permeable ion channel that acts as a nociceptor, sensing acute and inflammatory pain signals [34-36]. TRPA1 has already been shown to be activated by environmental irritants and oxidative- and thiol-reactive compounds, some of which are endogenously produced under oxidative stress conditions [37-39]. In particular, mutagenesis studies have identified that cysteine 414, cysteine 421, and cysteine 621 of human TRPA1 are targeted by electrophilic compounds such as mustard oil (MO) and cinnamaldehyde (CA) to activate TRPA1 [38,40]. On the other hand, non-electrophilic compounds like $\Delta 9$ -tetrahydrocannabinol, nicotine, and menthol also are also shown to activate TRPA1 via unknown mechanisms [37,41,42]. Moreover, currently used clinical drugs such as acetaminophen, dihydropyridines and general anaesthetics stimulate TRPA1, which may account for the clinical and adverse actions of these drugs [43-46]. Also, the antitumor drugs cisplatin (CIS) and oxaliplatin (OXA) activate TRPA1 via the generation of reactive oxygen species, which in turn may lead to peripheral neuropathy (PN) [47]. It is therefore noteworthy to investigate the effects of clinical drugs and experimental agents on the human TRPA1 channel. In the present study, we have shown that TRPA1 is expressed in primary cultured CAF cells from human PCa and PS30 cell line but not in PCa epithelial cells. Interestingly, we observed that TRPA1 activation by RES induced the expression (Figure 5C) and secretion (Figures 5A and B) of growth factors as HGF and VEGF. This RES-induced gene expression could be mediated by the activation of the estrogen receptor agonism and/or Calcineurin/NFAT pathway activated following an increase in the Ca^{2+} concentration. Indeed, it has been shown that resveratrol is a phytoestrogen and that it exhibits variable degrees of estrogen receptor agonism and the receptor mediates the transcription activation induced by RES in different cell systems [48]. As shown in the present work, RES induces the activation of TRPA1 leading to a Ca^{2+} entry. One of the possibilities of the transduction pathways induced by RES leading to gene expression is the activation of the Calcineurin/NFAT pathway elicited by an increase in the Ca^{2+} concentration induced via the activation of the TRPA1 channel. Indeed, we have shown that TRPA1 activation induces a nuclear translocation of NFATC3 leading to a specific gene expression (data under publication). As shown in figure 5, the RES-induced HGF and VEGF gene expression are inhibited by a TRPA1 inhibitor. These data suggest that the RES-induced gene expression in the PS30 cells could be mediated by calcium-dependent mechanisms including the Calcineurin/NFAT pathway.

Taken together, our data suggest an important impact of RES in TME through the activation of TRPA1 calcium-permeable channel and a subsequent growth factors secretion which might modulate cancer cells growth, migration and resistance to apoptosis. Indeed, our present data show that at high concentrations ($\geq 50\mu\text{M}$), RES induced apoptosis in PCa epithelial cells LNCaP (Figure 5D) while this apoptosis is strongly decreased when LNCaP cells are co-cultured with PS30 cells. These data suggest that PCa CAFs promote apoptosis resistance to RES treatments (Figure 5E and F) through paracrine secretion pathways. TRPA1 being involved in growth factors secretion such as HGF and VEGF, our results show that the ion channel participates actively to this crosstalk allowing apoptosis resistance against RES. Moreover, TRPA1 inhibition with HC-030031 or A-967079 re-establishes the apoptotic rate observed in LNCaP cells cultured alone. Consequently, the present work suggests that it is essential to study the effects of a molecule such as RES in the presence of the different cell components of the tumor microenvironment in order to accurately understand these effects on human prostate cancers.

Concerning the modulation of TRPA1 activity by RES, it has been shown that the mouse and rat ortholog of TRPA1 channels are inhibited by the polyphenol [21,22]. In the present work we show that RES also inhibits the wild type (WT) human TRPA1 (Figure 3F) but surprisingly, that it activates the TRPA1 expressed in human prostate cancer CAF cells (Figure 3). These data are of importance because if the TRPA1 ion channel expressed in mouse and rat is inhibited by RES, it could interfere on the epithelium-stroma crosstalk and reduce tumorigenesis in studies conducted in nude with subcutaneous injection of epithelial cancer cells alone. To take this aspect in consideration, human CAFs, such as PS30 cells, expressing a mutated TRPA1 ion channel activated by RES, should be co-injected with epithelial cancer cells to better mimic the human TME when considering the PCa cell tumorigenesis studies. Thus, *in vivo* models using only human epithelial tumor cells without human cancer-associated stromal cells can constitute a severe limit in the evaluation of RES or other molecules efficacy against prostate cancer.

In the present study, in order to better understand the discrepancy between wt-hTRPA1 (inhibited by RES) and the PCa CAF-expressed TRPA1 (activated by RES), we cloned and sequenced the entire cDNA sequence of the TRPA1 channel expressed in human PCa CAF cells. Compared to wt-hTRPA1, we observed 3 mutations in the N-terminal cytoplasmic domain of the channel (Figure 7). These mutations seem to be involved in the effects of the RES on CAF cells TRPA1. As mentioned above, according to the hTRPA1 SNP databases, two of the mutations (Y69C, K186N) were the SNP and one (K539R) a mutation. Amino

acids modifications (SNP or mutations) in the N-terminal domain of hTRPA1 are known to modulate the activation levels and sensitivity of the TRPA1 channel. Indeed, it has been described that the channels with Y69C and K186N substitutions exhibited high expression and sensitivity to TRPA1 agonists. In this report, a substitution in TRPA1 (Y69C) exhibited high expression/sensitivity to agonists [23]. These observations could explain the activation of TRPA1 expressed in human prostate CAFs while the wt-hTRPA1 is inhibited by the polyphenol. These data suggest also that, depending on the patients, differences in TRPA1 sequences could determine the potential efficacy of RES or other molecules used in PCa therapies. This shows the need for the establishment of a personalized medicine taking into account the patient's environment and proteins expression profiles. According to Morgan et al. (2015), approximately 130 single residues variants of TRPA1 are currently recorded in the populations (see summaries in NCBI single nucleotide polymorphism (SNP) database). The present data suggest also that the variations (mutations/SNP) in TRPA1 sequences could confer to the ion channel a high sensitivity to environment factors, promoting the progression of PCa via the secretion of growth factors by CAF. In addition, plausibly, there may be a relationship between the mutation of the TRPA1 channel and the occurrence and/or progression of PCa in men. Actually, there is no data studying the relationship between TRPA1 sequence modifications due to mutations/SNP and PCa. These interesting and important aspects need further investigations to propose preventive measures or to design new therapies targeting the tumor microenvironment interactions. In the future, the high-throughput sequencing and detection of mutations and/or SNPs could allow the adjustment of PCa treatments, such as the targeting of the CAF TRPA1 channel along with the use of RES or other molecules as anti-cancer agents to induce the apoptosis of the PCa cells, as suggested by the data presented in the present work.

In conclusion, we have shown that RES induces expression and secretion of growth factors such as HGF and VEGF through in the present work that a mutated TRPA1 ion channel expressed in human PCa CAF promotes apoptosis resistance of PCa cells. This work also shows the importance of taking into consideration the human tumour microenvironment in the efficacy studies of molecules such as RES in human PCa therapies which could be based on tumor-stroma paracrine interactions via the secretion of the growth factors and its modulation.

Acknowledgments

We would like to thank Dr A. Ogenesian, Department of Anesthesiology & Pain Medicine, University of Washington (Seattle, Washington, USA) for providing the PS30 cell line and E Richard for the technical assistance in the analysis of confocal microscopy images.

Competing financial interests

The authors declare no competing financial interests

REFERENCES

1. Jang M, Cai L, Udeani GO et al. Cancer chemopreventive activity of resveratrol, a natural product derived from grapes. *Science* 1997;275(5297):218-220.
2. Aziz MH, Nihal M, Fu VX, Jarrard DF, Ahmad N. Resveratrol-caused apoptosis of human prostate carcinoma LNCaP cells is mediated via modulation of phosphatidylinositol 3'-kinase/Akt pathway and Bcl-2 family proteins. *Mol Cancer Ther* 2006;5(5):1335-1341.
3. Benitez DA, Pozo-Guisado E, Alvarez-Barrientos A, Fernandez-Salguero PM, Castellon EA. Mechanisms involved in resveratrol-induced apoptosis and cell cycle arrest in prostate cancer-derived cell lines. *J Androl* 2007;28(2):282-293.
4. Fulda S, Debatin KM. Sensitization for tumor necrosis factor-related apoptosis-inducing ligand-induced apoptosis by the chemopreventive agent resveratrol. *Cancer Res* 2004;64(1):337-346.
5. Fulda S, Debatin KM. Sensitization for anticancer drug-induced apoptosis by the chemopreventive agent resveratrol. *Oncogene* 2004;23(40):6702-6711.
6. Jazirehi AR, Bonavida B. Resveratrol modifies the expression of apoptotic regulatory proteins and sensitizes non-Hodgkin's lymphoma and multiple myeloma cell lines to paclitaxel-induced apoptosis. *Mol Cancer Ther* 2004;3(1):71-84.
7. Liotta LA, Kohn EC. The microenvironment of the tumour-host interface. *Nature* 2001;411(6835):375-379.
8. Wernert N. The multiple roles of tumour stroma. *Virchows Arch* 1997;430(6):433-443.
9. Wernert N, Locherbach C, Wellmann A, Behrens P, Hugel A. Presence of genetic alterations in microdissected stroma of human colon and breast cancers. *Anticancer Res* 2001;21(4A):2259-2264.
10. Katz D. *The Release of Neural Transmitter Substances*. Liverpool University Press 1969.
11. Burgoyne RD. Mechanisms of catecholamine secretion from adrenal chromaffin cells. *J Physiol Pharmacol* 1995;46(3):273-283.
12. Neher E, Zucker RS. Multiple calcium-dependent processes related to secretion in bovine chromaffin cells. *Neuron* 1993;10(1):21-30.
13. Derouiche S, Warnier M, Mariot P et al. Bisphenol A stimulates human prostate cancer cell migration via remodelling of calcium signalling. *Springerplus* 2013;2(1):54.
14. Carpenter SL, Goldman J, Sherman AK et al. Clinical variables and *Staphylococcus aureus* virulence factors associated with venous thromboembolism in children. *Thromb Res* 2016;138:69-73.
15. Derouiche S, Mariot P, Warnier M et al. Activation of TRPA1 Channel by Antibacterial Agent Triclosan Induces VEGF Secretion in Human Prostate Cancer Stromal Cells. *Cancer Prev Res (Phila)* 2017.

16. Price DT, Rudner X, Michelotti GA, Schwinn DA. Immortalization of a human prostate stromal cell line using a recombinant retroviral approach. *J Urol* 2000;164(6):2145-2150.
17. Roudbaraki M, Lorsignol A, Langouche L, Callewaert G, Vankelecom H, Deneff C. Target cells of gamma3-melanocyte-stimulating hormone detected through intracellular Ca²⁺ responses in immature rat pituitary constitute a fraction of all main pituitary cell types, but mostly express multiple hormone phenotypes at the messenger ribonucleic acid level. Refractoriness to melanocortin-3 receptor blockade in the lactosomatotroph lineage. *Endocrinology* 1999;140(10):4874-4885.
18. Takayama H, LaRochelle WJ, Sharp R et al. Diverse tumorigenesis associated with aberrant development in mice overexpressing hepatocyte growth factor/scatter factor. *Proc Natl Acad Sci U S A* 1997;94(2):701-706.
19. Kopp RF, Leech CA, Roe MW. Resveratrol Interferes with Fura-2 Intracellular Calcium Measurements. *J Fluoresc* 2014;24(2):279-284.
20. Paudel RC, Kiviluoto S, Parys JB, Bultynck G. Resveratrol is not compatible with a Fura-2-based assay for measuring intracellular Ca(2)(+) signaling. *Biochem Biophys Res Commun* 2014;450(4):1626-1630.
21. Yu L, Wang S, Kogure Y, Yamamoto S, Noguchi K, Dai Y. Modulation of TRP channels by resveratrol and other stilbenoids. *Mol Pain* 2013;9:3.
22. Nalli M, Ortar G, Moriello AS, Morera E, Di Marzo V, De Petrocellis L. TRPA1 channels as targets for resveratrol and related stilbenoids. *Bioorg Med Chem Lett* 2016;26(3):899-902.
23. Morgan K, Sadofsky LR, Morice AH. Genetic variants affecting human TRPA1 or TRPM8 structure can be classified in vitro as 'well expressed', 'poorly expressed' or 'salvageable'. *Biosci Rep* 2015;35(5).
24. Shih A, Zhang S, Cao HJ et al. Inhibitory effect of epidermal growth factor on resveratrol-induced apoptosis in prostate cancer cells is mediated by protein kinase C-alpha. *Mol Cancer Ther* 2004;3(11):1355-1364.
25. Lin HY, Shih A, Davis FB et al. Resveratrol induced serine phosphorylation of p53 causes apoptosis in a mutant p53 prostate cancer cell line. *J Urol* 2002;168(2):748-755.
26. Selvaraj S, Sun Y, Sukumaran P, Singh BB. Resveratrol activates autophagic cell death in prostate cancer cells via downregulation of STIM1 and the mTOR pathway. *Mol Carcinog* 2016;55(5):818-831.
27. Orihuela-Campos RC, Tamaki N, Mukai R et al. Biological impacts of resveratrol, quercetin, and N-acetylcysteine on oxidative stress in human gingival fibroblasts. *J Clin Biochem Nutr* 2015;56(3):220-227.
28. Giovannelli L, Pitozzi V, Jacomelli M et al. Protective effects of resveratrol against senescence-associated changes in cultured human fibroblasts. *J Gerontol A Biol Sci Med Sci* 2011;66(1):9-18.
29. Olson ER, Naugle JE, Zhang X, Bomser JA, Meszaros JG. Inhibition of cardiac fibroblast proliferation and myofibroblast differentiation by resveratrol. *Am J Physiol Heart Circ Physiol* 2005;288(3):H1131-1138.
30. Walle T, Hsieh F, DeLegge MH, Oatis JE, Jr., Walle UK. High absorption but very low bioavailability of oral resveratrol in humans. *Drug Metab Dispos* 2004;32(12):1377-1382.
31. Walle T. Bioavailability of resveratrol. *Ann N Y Acad Sci* 2011;1215:9-15.
32. Miksits M, Wlcek K, Svoboda M et al. Antitumor activity of resveratrol and its sulfated metabolites against human breast cancer cells. *Planta Med* 2009;75(11):1227-1230.

33. Andreadi C, Britton RG, Patel KR, Brown K. Resveratrol-sulfates provide an intracellular reservoir for generation of parent resveratrol, which induces autophagy in cancer cells. *Autophagy* 2014;10(3):524-525.
34. Bautista DM, Jordt SE, Nikai T et al. TRPA1 mediates the inflammatory actions of environmental irritants and proalgesic agents. *Cell* 2006;124(6):1269-1282.
35. Kwan KY, Allchorne AJ, Vollrath MA et al. TRPA1 contributes to cold, mechanical, and chemical nociception but is not essential for hair-cell transduction. *Neuron* 2006;50(2):277-289.
36. Obata K, Katsura H, Mizushima T et al. TRPA1 induced in sensory neurons contributes to cold hyperalgesia after inflammation and nerve injury. *J Clin Invest* 2005;115(9):2393-2401.
37. Jordt SE, Bautista DM, Chuang HH et al. Mustard oils and cannabinoids excite sensory nerve fibres through the TRP channel ANKTM1. *Nature* 2004;427(6971):260-265.
38. Macpherson LJ, Dubin AE, Evans MJ et al. Noxious compounds activate TRPA1 ion channels through covalent modification of cysteines. *Nature* 2007;445(7127):541-545.
39. Story GM, Peier AM, Reeve AJ et al. ANKTM1, a TRP-like channel expressed in nociceptive neurons, is activated by cold temperatures. *Cell* 2003;112(6):819-829.
40. Hinman A, Chuang HH, Bautista DM, Julius D. TRP channel activation by reversible covalent modification. *Proc Natl Acad Sci U S A* 2006;103(51):19564-19568.
41. Karashima Y, Damann N, Prenen J et al. Bimodal action of menthol on the transient receptor potential channel TRPA1. *J Neurosci* 2007;27(37):9874-9884.
42. Talavera K, Gees M, Karashima Y et al. Nicotine activates the chemosensory cation channel TRPA1. *Nat Neurosci* 2009;12(10):1293-1299.
43. Andersson DA, Gentry C, Alenmyr L et al. TRPA1 mediates spinal antinociception induced by acetaminophen and the cannabinoid Delta(9)-tetrahydrocannabinol. *Nat Commun* 2011;2:551.
44. Fajardo O, Meseguer V, Belmonte C, Viana F. TRPA1 channels: novel targets of 1,4-dihydropyridines. *Channels (Austin)* 2008;2(6):429-438.
45. Matta JA, Cornett PM, Miyares RL, Abe K, Sahibzada N, Ahern GP. General anesthetics activate a nociceptive ion channel to enhance pain and inflammation. *Proc Natl Acad Sci U S A* 2008;105(25):8784-8789.
46. Nassini R, Materazzi S, Andre E et al. Acetaminophen, via its reactive metabolite N-acetyl-p-benzo-quinoneimine and transient receptor potential ankyrin-1 stimulation, causes neurogenic inflammation in the airways and other tissues in rodents. *FASEB J* 2010;24(12):4904-4916.
47. Nassini R, Gees M, Harrison S et al. Oxaliplatin elicits mechanical and cold allodynia in rodents via TRPA1 receptor stimulation. *Pain* 2011;152(7):1621-1631.
48. Gehm BD, McAndrews JM, Chien PY, Jameson JL. Resveratrol, a polyphenolic compound found in grapes and wine, is an agonist for the estrogen receptor. *Proc Natl Acad Sci U S A* 1997;94(25):14138-14143.

LEGENDS TO FIGURES

Figure 1: Effects of RES on human prostate cancer (PCa)-associated fibroblasts (CAF)

(A) Microscope examination of PS30 cells treated with different concentrations of RES. PS30 cells were treated with RES at different concentrations (5, 25 and 50 μM) for 72h in RPMI medium containing 10% FCS. (B) Western blotting experiments showing the effects of RES on CAF cells markers (α -actin and vimentin) expression. Densitometric quantification for normalized α -actin and vimentin relative to calnexin as an internal standard is shown. Cells were treated with RES (0,1 to 100 μM , 72h) and then processed for Western blotting experiments as described in Materials and Methods section. (C) Cell growth was measured by MTS/PMS assay on prostate CAFs (PS30) treated with RES at different concentrations (0,1 to 100 μM , 72h). (D) The impact of RES on apoptosis was assessed by a Hoechst staining technique. PS30 cells were treated for 72h with RES (0,1 to 100 μM) and then their nuclei were stained as described in Materials and Methods section. Apoptotic cells were then counted under a fluorescence microscope. (E, F) Prostate cancer CAFs (10^5 cells/well) were incubated in triplicate with RES (10, 25 and 50 μM) or BAPTA/AM (5 μM) in RPMI medium containing 0.5% FCS for 24h. Then, ELISA assays were realized on conditioned media in order to determine the variation in the secretion of HGF (E) and VEGF (F). BAPTA/AM (5 μM) was used in order to determine if the basal secretion process was Ca^{2+} -dependent. The data (C-F) represent the mean \pm S.E. from three experiments performed in triplicate. All experiments were performed at least 3 times in 3 independent cell cultures and representative figures are shown. *- $p < 0.05$, ** - $p < 0.001$,

Figure 2: RES induces Ca^{2+} entry in prostate CAFs

(A) Impact of RES on the cytosolic Ca^{2+} concentration was studied in PS30 cells by Ca^{2+} imaging technique by using ratiometric Fura-2 as a fluorescent Ca^{2+} dye. RES was applied at different concentrations on the cells. Ca^{2+} concentration measurements are represented by the F340/F380 ratio. (B) Dose-response curve of RES effects on cytosolic Ca^{2+} concentrations in PS30 cells ($\text{EC}_{50} = 6.49\mu\text{M}$). (C) Anti-parallel variations of Fura-2 fluorescence emission at 510 nm after excitation at 340 nm (F340) and at 380 nm (F380) when RES (25 μM) was applied. The corresponding F340/F380 ratio is presented (D) showing the impact of RES on cytosolic calcium concentrations. (E) Calcium imaging experiments performed on PS30 cells using Fluo-4 as a fluorescent Ca^{2+} dye (excitation, 470 nm; emission, 525 nm) showing Ca^{2+} response induced by RES (25 μM). (F) To determine whether Ca^{2+} responses induced by RES were the result of a mobilization of the intracellular Ca^{2+} stores or a Ca^{2+} entry, RES (25 μM) was applied to PS30 cells in Ca^{2+} -free HBSS medium (0 Ca^{2+}) and in HBSS containing 2mM Ca^{2+} (2 Ca^{2+}) medium during Ca^{2+} imaging experiments using Fura-2 as a calcium probe. Ca^{2+} imaging experiments are represented as mean \pm S.E of 40 cells. All experiments were performed at least 3 times in 3 independent cell cultures and representative figures are shown.

Figure 3: Resveratrol activates TRPA1 ion channel in prostate CAFs

Ca^{2+} imaging experiments showing the involvement of TRPA1 ion channel in RES-induced calcium entry in PS30 cell line (A, B) and in primary cultured PCa CAF (C). In the latter cells,

the application of RES induced a calcium response and failed to inhibit the AITC-induced activation of TRPA1 channel (**D**). The involvement of the TRPA1 channel is shown either by using the TRPA1 inhibitor (HC-030031, 50 μ M) (**A**, **B**, **C**) or by the transfection of the cells with siTRPA1 in order to down-regulate the TRPA1 protein expression (**E**). TRPA1 involvement in the Ca²⁺ entry induced by RES was confirmed by Ca²⁺ imaging studies with the application of RES (25 μ M) on PS30 cells transfected with siRNA directed against TRPA1 mRNA (siTRPA1, 25 nM for 48h) and siCTL (25 nM, 48h). The TRPA1 inhibitor (HC-030031, 50 μ M) is applied either during the Ca²⁺ response (**A**) or before the application of RES (**B**, **C**). (**F**) In calcium imaging experiments, the effects of AITC (30 μ M) on intracellular calcium concentration was studied in the presence and absence of RES (25 μ M) in HEK293 cells transiently transfected with human wt-TRPA1 expression vector (HEK-wtTRPA1). Ca²⁺ imaging experiments are represented as mean \pm S.E of 40 cells. All experiments were performed at least 3 times in 3 independent cell cultures and representative figures are shown.

Figure 4: Human prostate CAFs express functional TRPA1 channel

(**A**) Semi-quantitative RT-PCR experiments were performed to study the expression of TRPA1 (510 bp) mRNA using primers TRPA1-1 (see Table 1 for sequences) in PS30 cells, PrCSC (primary cultured CAF), epithelial prostate cancer cell lines (LNCaP, DU145 and PC-3) and in hPEC (primary cultured prostate cancer epithelial cells). Protein expression of TRPA1 and myofibroblasts markers: α -actin and vimentin was studied by western blot in PS30 cells and PrCSC (**B**) and by immunofluorescence experiments in PS30 cells (**C**). (**D**) TRPA1 activation by AITC ranging from 500 nM to 30 μ M monitored with Ca²⁺ imaging technique in PS30 cells. (**E**) Dose–response curve showing the amplitude of calcium response (TRPA1 activation) as a function of AITC concentrations (EC₅₀ = 5,19 μ M).

Figure 5: RES modulates growth factors secretion in prostate CAFs and participates to the epithelium-stroma crosstalk through TRPA1 activation

In order to study whether TRPA1 was involved in the Ca²⁺-dependent secretion induced by RES, PS30 cells were treated with RES at different concentrations (10, 25 and 50 μ M for 24h) in presence or absence of a TRPA1 inhibitor, (HC-030031 (50 μ M). Then, the conditioned media were processed by ELISA assays in order to determine the secretion rates of HGF (**A**) and VEGF (**B**). (**C**) Study of HGF and VEGF expression by RT-PCR experiments performed on the mRNA of PS30 cells treated with RES at different concentrations (1, 10 and 25 μ M) in presence or absence of HC-030031 (50 μ M) for 48h. HGF and VEGF expression rates in each sample are normalized by densitometry studies using β -Actin expression as internal standard. (**D**) The impact of RES on LNCaP cells apoptosis was assessed by Hoechst staining experiments. LNCaP cells treated for 72h with RES (0,1 to 100 μ M) were stained with Hoechst 33342 dye (4 μ g/mL) and apoptotic cells (with nuclear condensation) were identified and counted under a fluorescence microscope as described in Materials and Methods. (**E**) Schematic representation of the co-cultures between LNCaP cells and PS30 cells separated each other by an insert with pores of 0.4 μ m diameter pores. (**F**) The co-cultures of LNCaP and PS30 cells were realized as shown in (E). The two cell types were then treated with RES (50 μ M, 72h) in presence or absence of HC-030031 (50 μ M) in order to determine their impact on CAFs and the involvement of TRPA1 channel on the LNCaP apoptosis induced by RES (50 μ M). The rate of LNCaP apoptosis was determined by Hoechst staining experiments as described for (D). Data represent the mean \pm S.E. from three experiments performed in triplicate. * p<0.001 compared with non-treated controls or with appropriate controls as shown on figures.

Figure 6: Inhibition of TRPA1 channel expressed by PCa CAFs restores apoptosis induced by RES in LNCaP cells

LNCaP and PS30 cells were co-cultured as previously described and the two cell types were treated with RES (50 μ M, 72h) in presence or absence of HC-030031 (50 μ M) in order to determine the impact of CAF and the TRPA1 channel on the RES (50 μ M)-induced apoptosis rate of LNCaP cells. (A) TUNEL assay realized on LNCaP and PS30 cells treated with RES (50 μ M) for 72h. All nuclei are labeled in blue with DAPI and apoptotic nuclei are labeled in pink (blue + red); Apoptosis was quantified by apoptotic nuclei counting (labeled in pink) as described in material and methods (B) ($n = 6$). The data represent the mean \pm S.E. from three experiments performed in triplicate.* $p < 0.001$ compared with non-treated controls or with appropriate controls as shown on figures.

Figure 7: RES activates a mutated TRPA1 channel expressed in PS30 cells

(A) The entire cDNA sequence for TRPA1 expressed in PS30 cells was cloned by RT-PCR and sequenced as described in Materials and Methods. The resulting sequence was translated into the predicted amino acid sequence of the PS30 TRPA1 and aligned with the amino acid sequence of full length wild type hTRPA1 (accession number NM_007332). Boxed sequences represent mutated amino acids in PS30 TRPA1 sequence. As the mutations are observed in the N-terminus sequence of the PS30 TRPA1, only the sequence alignments of this region are shown. (B) Schematic localization of the mutations in TRPA1 channel expressed in PS30 cells comprising 2 SNPs (K186N and Y69C) and one mutation (K539R) in the N-terminal region. (C) In order to characterize the importance of TRPA1 channel expressed in PS30 cells in the Ca²⁺ response induced by RES (25 μ M), the latter cells were transfected with the expression vector for wt-TRPA1, a channel inhibited by RES and then Ca²⁺ imaging experiments were then performed to study the effects of RES and AITC in PS30 transfected cells.