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Activation of TRPA1 Channel by Antibacterial Agent Triclosan Induces VEGF Secretion in Human Prostate Cancer Stromal Cells

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Abstract

Accruing evidence indicates that exposure to environmental compounds may adversely impact human health and promote carcinogenesis. Triclosan (TCS), an antimicrobial agent widely used as a preservative in personal care products, has been shown to act as an endocrine disruptor in hormone-dependent tissues. Here we demonstrate a new molecular mechanism by which TCS stimulates the secretion by human prostate cancer stromal cells of Vascular Endothelial Growth Factor (VEGF), a factor known to promote tumour growth. This mechanism involves an increase in intracellular calcium levels due to the direct activation of a membrane ion channel. Using calcium imaging and electrophysiology techniques, we show for the first time that environmentally relevant concentrations of TCS activate a cation channel of the TRP family, TRPA1 (Transient Receptor Potential Ankirin 1), in primary cultured human prostate cancer stromal cells. The TCS-induced TRPA1 activation increased basal calcium in stromal cells and stimulated the secretion of VEGF and epithelial cells proliferation. Interestingly, immunofluorescence labeling performed on formalin-fixed paraffin-embedded prostate tissues showed an exclusive expression of the TRPA1 channel in prostate cancer stromal cells. Our data demonstrate an impact of the environmental factor TCS on the tumor microenvironment interactions, by activating a tumor stroma-specific TRPA1 ion channel.

INTRODUCTION

Prostate cancer is among the most frequent malignancies in industrialized nations and continuous research efforts are undertaken in order to better understand its development and progression. Androgen dependence of this tumour is long known (1-3) but epidemiological studies suggest that factors from Western life style also contribute to its development (4, 5).

Epithelial stromal interactions are considered important for prostate cancer development and progression (6, 7). Carcinomas in general are composed of two interdependent components: the neoplastic epithelial cells and the supporting tumour stroma, which plays decisive roles in pivotal processes such as tumour proliferation, vascularization and invasion (8-10) *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: the 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 (11-14), including breast and prostate cancers (9, 15-17). Modulation of these stromal factors secretion might impact the initiation or progression of prostate cancers. In addition to the epithelial-derived and inflammatory factors, environmental molecules might target the stromal cells to modify their physiology including their secretion capacity. One of the mechanisms involved in the effects of the environmental compounds is the modification of the cellular calcium homeostasis (18). It is well established that Ca^{2+} responses to extracellular stimuli can lead to rapid secretion of growth factors and hormones through exocytosis (19).

Accumulating data indicate that exposure to environmental compounds may adversely impact human health. Triclosan (TCS; 2,4,4'-trichloro-2'-hydroxydiphenyl ether; a chlorophenol) is an antimicrobial agent widely used as preservative in toothpastes, soaps, shampoos, and cosmetics at concentrations up to 0,3% or 10 mM (20). TCS is known to be a highly toxic chemical for aquatic flora and fauna (21) and thus has been included in the probable list of endocrine disruptors (ED). Triclosan is also detected in human blood plasma (22), breast milk (at concentration of 1-10 μM) (23) and urine (24) and daily exposure to TCS of breast tissue was estimated to be of 5.5 μM (25, 26). At such levels, TCS has been shown to modulate the growth of mammary cancer cells (27) but its potential effects are not known on human prostate cancer epithelial and stromal cells. The mode of action of TCS as an ED is controversial and it was recently showed that TCS could exert its effects *via* several mechanisms including the modulation of Androgen Receptor (28), the modulation of the activity of an adenylyl cyclase enzyme (29) and, recently, the activation of calcium signalling

(25). The latter effect of TCS is of importance because it is well established that in most cell types, increase in cell calcium (Ca^{2+}) concentrations is associated to cell growth and/or secretory responses (30).

As epithelial stromal interactions play a pivotal role in the progression of prostate cancers, environmental factors like TCS may impact these interactions to favour this progression. The present report was designed to determine the effects of TCS on both epithelial and stromal cells derived from prostate cancer tissues. Our work focused on the effects of this environmental factor on calcium signalling, an important parameter controlling the secretion of the mitogenic and/or angiogenic factors by stromal cells known to trigger epithelial and endothelial cells development.

Using a combination of immuno-staining, calcium imaging and electrophysiology techniques, we show here for the first time that the widely used antimicrobial TCS induces the activation of TRPA1 calcium permeable channels and the subsequent release of Vascular Endothelial Growth Factor (VEGF) in human prostate cancer stromal cells. We also show the exclusive expression of the TRPA1 protein in stromal cells of human prostate cancer tissue. TCS could thus have a significant impact on prostate carcinogenesis through the activation of TRPA1 channels and subsequent release of VEGF.

Materials and Methods

Chemicals

All chemicals were from Sigma-Aldrich. Triclosan (Irgasan) and triclocarban were dissolved in DMSO, conserved at -20°C and diluted at the desired concentration on the day of the experiments.

Cell lines

LNCaP, PC-3 and DU145 prostate cancer cell lines and HEK293 (human embryonic kidney-derived 293) cell line were obtained from the American Type Culture Collection (ATCC, Manassas, VA, USA) and cultured as described by Gackiere *et al.* (31).

Tissue specimens and primary cell cultures

Human PCa biopsies were obtained from consenting patients following the 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'. A portion of prostate tissue suspicious for 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 in multiple minute cubicles, placed on a plastic surface, and grown in Phenol red-free RPMI 1640 containing charcoal-stripped Fetal Calf Serum (FCS) (CS-RPMI) containing 1 nM DHT for the growth of Prostate cancer Stromal Cells (PrSC) and in keratinocyte medium supplemented with 50 mg/ml bovine pituitary extract, 5 ng/ml epidermal growth factor (EGF), 100 mg/ml streptomycin, and 100 U/ml penicillin (Life Technologies, Inc., Gaithersburg, MD) and 2% FCS for the epithelial cells growth. PrSC cultures were validated by immunofluorescence using anti- α -actin and mouse anti-Vimentin (Dako, France) antibodies.

Cell transfections and RT-PCR analysis

PrSC were transfected overnight with 25 nM of control siRNA (targeting Luciferase mRNA) (Eurogentec, Belgium) or a siRNA raised against TRPA1 mRNA. (siTRPA1-1, target sequence 5'-GGUGGGAUGUUAUCCAUA(dTdT)-3' or siTRPA1-2: 5'-GAAGGACGCUCUCCACUUA(dTdT)-3') as previously described (32) and used 48h post-transfection. The efficiency of the siRNAs was validated by RT-PCR and by western blot studies (shown for siTRPA1-1 in Fig. 2D). RT-PCR experiments and HEK293 stably transfected by either hTRPA1 coding sequence (HEK-hTRPA1) or by pcDNA3 vector (HEK-vector) were realized as described earlier (32). The PCR primers (hTRPA1: 5'-AGTGGCAATGTGGAGCAA-3' and 5'-TCTGATCCACTTTGCGTA -3'; β -actin : 5'-CAGAGCAAGAGAGGCATCCT-3' and 5'-GTTGAAGGTCTCAAACATGATC-3' used in this study were designed on the basis of established GenBank sequences and synthesized by Invitrogen (Carlsbad, CA, USA). The amplified PCR products were of 510 and 212 bp respectively.

Immunofluorescence studies

The protein expression studies of the ion channels in PCa cells and tissues was determined by indirect immunofluorescence analysis performed on acetone-fixed cells and formalin-fixed paraffin embedded (FFPE) tissues as previously described (32) and analysed by confocal microscopy (Zeiss LSM 780; acquisition parameters: objective 40x/1.3; thickness of confocal slide, 1 μ m).

Western Blot Assay

PrSC and HEK293 cells were cultured at 80% of confluence and total proteins extracted. Ten to forty micrograms of each sample were analysed by western-blotting using hTRPA1 (Alomone Labs, 1/500e) β -actin (Sigma-Aldrich, 1/2000e) and Calnexin (Santa Cruz Biotechnology, 1/200e) antibodies and ECL technique as described previously (32).

Cytosolic free Ca^{2+} concentration ($[Ca^{2+}]_i$) Measurements

Cells were grown on glass coverslips and $[Ca^{2+}]_i$ imaging experiments were performed in HBSS (Hanks Balanced Salt Solution in mM; 142 NaCl, 5.6 KCl, 1 MgCl₂, 2 M CaCl₂, 0,34 Na₂PO₄, 0,44 KH₂PO₄, 10 HEPES, 5.6 glucose and buffered to pH 7.4) as previously described (32) using Fura-2 AM as calcium dye. To represent the variation of the $[Ca^{2+}]_i$, the fluorescence intensity ratio represented by F340/F380 was used as an indicator of changes in cytosolic Ca^{2+} concentration. Each experiment was repeated at least 4 times in duplicate on different cell cultures on a field of 35–45 cells and representative experiments performed on 60-90 cells as mean \pm s.e are presented.

Electrophysiological studies

Whole-cell patch-clamp recordings were performed using a RK-300 patch-clamp amplifier (Biologic, Grenoble, France) as previously described (31). Bath medium used for whole-cell experiments consisted in HBSS supplemented with 10 mM TEA to block potassium currents. Recording pipettes were filled with a solution containing (in mM) 140 KGluconate, 10 NaCl, 10 HEPES, 2 MgCl₂, with 1 EGTA. Osmolarity and pH were adjusted to 290 mOsm.l⁻¹ and 7.2, respectively.

Preparation of PrSC conditioned medium and cell growth studies

Primary cultured stromal cells (5×10^5 cells per condition) were cultured in CS-RPMI supplemented with 0.1% FCS with or without TCS for 3 days. Culture supernatants were collected, centrifuged and then incubated with PC-3 cells for the cell growth studies. PC-3 cells seeded in 96-well plates (3000 cells/well) were cultured in RPMI/10% FCS for 24h. The medium was then replaced by 150 μ l of PrSC conditioned medium and incubated for 3 days. The control PC3 cells were cultured in CS-RPMI supplemented with 0.1% FCS for 3 days with or without TCS. Cell growth was estimated using the commercial assay MTS/PMS kit as previously described (32).

ELISA for VEGF

PrSC cells were incubated in CS-RPMI supplemented with 0.1 % FCS with or without TCS for 3 days into 12-well plates. The supernatants of the cell cultures were then withdrawn, centrifuged and a VEGF immunoassay kit (Abcam, Paris, France) was used for VEGF detection according to the manufacturer's instructions.

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

Triclosan induces calcium signalling in human prostate cancer stromal cells (PrSC)

We first assessed the effects of Triclosan (TCS) and its structural analog, Triclocarban (TCC) on prostate cancer stromal cells. The PrSC primary culture were first characterized for the expression of stromal cells markers by immunofluorescence studies using antibodies raised against α -actin and vimentin. Seven different cultures originated from 7 patients were included in this study. We observed that the proportion of cells expressing α -actin and/or vimentin varied between each primary cell culture. Globally, the PrSC cultures were divided in two categories, the first one in which 90% of cells expressed vimentin (fibroblasts) and the remaining 10% expressed α -actin (smooth muscle cells) (Fig. 1C, insert) and the other one in which almost 100% of cells expressed both vimentin and α -actin (myofibroblasts) (Fig. 1D, insert) suggesting the presence of only stromal cells in our cultures.

On these characterized stromal cells, by calcium imaging, we studied the effects of TCS and its structural analog, TCC (see chemical structures in inserts in Fig. 1A and 1B), on cytosolic free calcium concentration ($[Ca^{2+}]_i$). The $[Ca^{2+}]_i$ was continuously monitored as a fluorescence ratio F340/F380 using the calcium probe Fura-2/AM in a normal HBSS medium containing 2 mM $CaCl_2$ and then, at the time indicated, TCS or TCC was applied to the cells. In these experiments, TCS induced a rapid and dose-dependent increase in $[Ca^{2+}]_i$ in PrSC (Fig. 1A). These observations suggest that TCS is able to induce either a calcium entry and/or a Ca^{2+} release from intracellular stores. In the same experimental conditions, TCC failed to elicit any significant modulation of basal $[Ca^{2+}]_i$ in PrSC (Fig. 1B). Two types of TCS-

induced calcium increase were observed with regard to the kinetics and amplitudes (Fig. 1C and 1D). TCS induced an initial rapid and transient first phase followed by a sustained plateau phase characterized by either a low (Fig. 1C) or a high amplitude (Fig. 1D). The combined calcium imaging and immunofluorescence studies using α -actin and vimentin antibodies suggest that these two different types of calcium response are correlated with the preponderance of myofibroblasts for the high-amplitude plateau phase (Fig. 1D) and with the preponderance of fibroblasts for the low-amplitude plateau phase (Fig. 1C) in stromal cells populations. This robust effect of TCS on calcium entry was observed on PrSC from 6 out of 7 cancer tissues.

The origin of the TCS-induced calcium increase in human prostate stromal cells

In calcium imaging experiments, cells were challenged with TCS in a Ca^{2+} -free HBSS medium (Fig. 1E) followed by the addition of 2 mM calcium still in the presence of TCS. In the absence of external calcium, TCS induced a slight calcium response due to a mobilization of calcium from intracellular pools, through the activation of Type I Ryanodine Receptors (RyRs) as previously described in mouse skeletal myotubes (25). Interestingly, as shown in Figure 1E, when calcium was added in the external buffer, TCS induced a rapid and high amplitude calcium entry in human PrSC *via* the plasma membrane calcium channels. The same experiments performed on human prostate cancer epithelial cells (hPEC) showed that TCS only induced a calcium mobilization without promoting a calcium entry in these cells (Fig. 1F). We therefore undertook experiments in order to identify the calcium permeable membrane channels involved in the effects of TCS in primary cultured PrSC.

Involvement of TRPA1 in TCS-induced calcium response

To investigate the membrane ion channels involved in TCS-induced calcium entry, we used an extensive pharmacology comprising blockers of voltage-dependent, non voltage-dependent calcium channels and potassium channels ([Supplementary Fig. S1A](#)). Surprisingly, many tested blockers (inhibitors of L-Type voltage-dependent calcium channels and non voltage-dependent calcium channels (TRP)) were able to elicit an increase in $[\text{Ca}^{2+}]_i$ somehow similar to the one induced by TCS ([Supplementary Fig. S1B and S1C](#)). According to the published data, all these compounds were activators of a unique ion channel, the non voltage-dependent TRPA1 calcium channel (33-37). We thus assessed whether TRPA1 could be the ion channel activated by TCS in human PrSC.

We first used ruthenium red (RR) as a non selective inhibitor of TRPA1 channel. As shown in Figure 2A, the TCS-induced calcium entry was dose-dependently blocked by RR. Moreover, the calcium increase observed by application of flufenamic acid, was also inhibited by ruthenium red (Fig. 2B), suggesting the involvement of TRPA1 in flufenamic acid effects in these cells. We next used a selective inhibitor of TRPA1, the compound HC-030031, which showed a dose-dependent inhibitory effect on the calcium response to TCS, reaching a maximum of inhibition for a concentration of 200 μ M (Fig. 2C and Supplementary Fig. S2A-S2F). The involvement of TRPA1 channel in the TCS-induced calcium response was confirmed by the use of AP-18 and A967079, two other inhibitors of TRPA1 channel. (Supplementary Fig. S3A-S2F). In these experiments, we also observed a significant decrease in basal calcium concentration after addition of HC-030031 (200 μ M) and RR (50 μ M) (Fig. 2B and 2C). These observations suggest that TRPA1 is involved in setting the basal calcium concentration in PrSC. To confirm these results, we also used siRNAs targeting TRPA1 (siTRPA1) to down-regulate the TRPA1 protein. Using RT-PCR and western blot techniques, we first showed that the siTRPA1 transfection (25 nM) of stromal cells induced a significant down-regulation of the TRPA1 mRNA and protein in these cells (Fig. 2D). We then observed in calcium experiments that the TCS-induced calcium entry was almost completely blocked in siTRPA1-transfected cells (Fig. 2E), leading to the conclusion that TRPA1 is the main ion channel involved in TCS-induced calcium entry. To confirm the functional TRPA1 expression in PCa stromal cells, the effects of a known TRPA1 agonist (Allyl Isothiocyanate, AITC) on cytosolic free calcium concentration in PrSC. As shown in Figure 2F, AITC (30 μ M) induced a calcium increase which was inhibited by HC-030031 (200 μ M).

We further investigated the potential activation of TRPA1 by TCS in PrSC by electrophysiological approaches using whole-cell patch clamp techniques. A voltage ramp of -100 to +120 mV (A voltage ramp of -80 to +80 mV) was applied and the resulting currents were recorded in the absence (CTL) or in the presence of TCS in the extracellular medium. As shown in Figures 3A-3D, TCS induced within few seconds after the onset of perfusion a large increase in membrane conductance at all membrane potentials. TCS-induced membrane current displayed a slight outward rectification and reversed around 0 mV. This TCS-induced current was reversible when cells were washed with TCS-free HBSS and was inhibited by the addition of HC-030031 (50 μ M). The action of TCS was similar to that of AITC which was able to activate TRPA1 current in PrSC, an effect also inhibited by HC-030031 (50 μ M) (Fig. 3E and 3F). To confirm the implication of TRPA1 in TCS-induced current, the effects of TCS were studied in HEK-hTRPA1 cells stably expressing the human form of TRPA1. In these

cells, both TCS (10 μ M) and AITC (50 μ M) reversibly increased membrane conductance, as in stromal cells, whereas they were ineffective in vector-transfected cells (HEK-vector cells) (Fig. 4A-D). Moreover, TCS- and AITC-induced currents were also inhibited by HC-030031 (50 μ M). These data clearly show that the antibacterial TCS induces the activation of hTRPA1.

Expression of TRPA1 channel in the human prostate cancer

We further studied the expression of TRPA1 channel in PrSC and human PCa epithelial cells. First, by RT-PCR analysis, we observed an absence of TRPA1 expression in epithelial cells while stromal cells obtained from different PCa tissues all expressed the mRNA for TRPA1 (Fig. 5A). TRPA1 expression was also confirmed in HEK-hTRPA1 cells by western-blot experiments. As shown in Figure 5B, the antibody revealed a band of expected size (~130 kDa) and a band of higher molecular weight which could represent a glycosylated form of TRPA1 protein. The same antibody was then used in western blot experiments performed on different prostate cancer epithelial cell lines (LNCaP and PC-3), primary cultured epithelial cells (hPEC) and stromal cells of different patients (PrSC) used in the present study. As shown in Figure 5C, the TRPA1 proteins were only present in protein extracts from stromal cells. This is in agreement with our results showing a stimulation of an HC-030031-sensitive calcium entry by TCS in stromal but not in epithelial cells. We further studied by immunofluorescence staining the expression of TRPA1 channel protein in 7 different non cancer (BPH) and cancer (PCa) tissues using formalin-fixed, paraffin-embedded tissues. As α -actin (smooth muscle actin) expression was restricted to the stroma, the positive staining for α -actin was used to discriminate between the epithelium and the stroma. Co-immunolabeling for TRPA1 (in green) and α -actin (in red) showed overlapping staining in prostate cancer regions showing a preferential expression of TRPA1 protein in stromal cells of the cancer regions (Fig. 5D and 5E) and in the primary culture stromal cells (Fig. 5F). The same co-immunolabeling experiments performed in non cancer (BPH) regions did not reveal any staining for TRPA1 protein neither in epithelial cells nor in stromal cells (Fig. 5H) whereas α -actin (in red) was clearly expressed in stromal cells (Fig. 5G and 5H). A control immunofluorescence experiment is presented (Fig. 5I) where the primary antibody (anti-TRPA1) was omitted.

These data clearly show the preferential expression of TRPA1 in prostate cancer stromal cells. To extend these data to other cancers, we studied the expression of TRPA1 in 3 breast non

cancer and cancer tissues. As shown in Fig. 5J-5L, TRPA1 protein is increased in cancer regions of breast tissues. These data suggest a common feature of TRPA1 expression in cancer and that the effects of TCS on TRPA1 channels could be of importance in cancer progression by inducing calcium entry and a possible secretion of factors necessary for the epithelium-stroma interactions.

Triclosan induces vascular endothelial growth factor (VEGF) secretion by stromal cells

To assess the influence of TCS on epithelial-stromal interactions, we examined the impact of TCS-pretreatments of PrSC on the proliferation of PC-3 PCa epithelial cells. In this context, we observed that when PrSC cells were cultured in CS-RPMI supplemented with 10% FCS, the PrSC-conditioned media stimulated an increase in proliferation of PC-3 cells. When PrSC were cultured in CS-RPMI with 0.1% FCS, the PrSC-conditioned media failed to stimulate the proliferation of PC-3 cells. Interestingly, in this low serum condition, the conditioned media from TCS-pretreated PrSC induced an increase in PC-3 cells growth (Fig. 6A) whereas in high serum condition (10% FCS), the conditioned media of TCS-treated PrSC induced only a faint increase of the PC-3 cell proliferation. We then carried out ELISA measurements of growth factors secretion in CTL and TCS-treated conditioned media of PrSC. Interestingly, the incubation of stromal cells with TCS (1-10 μ M) clearly induced an increase in VEGF secretion (Fig. 6B). In addition, TCS-induced VEGF secretion was inhibited by the TRPA1 inhibitor HC-030031 suggesting the involvement of TRPA1 channel in the effects of TCS on VEGF secretion by prostate stromal cells. In these experiments, the TRPA1 inhibitor HC-030031 showed a dose-dependent inhibitory effect on the TCS-induced VEGF secretion reaching a maximum of inhibition for a concentration of 200 μ M (Fig. 6B and Supplementary Fig. S4). The involvement of TRPA1 channel in the TCS-induced VEGF secretion was confirmed by the use of AP-18 and A967079, two other inhibitors of TRPA1 channel. (Supplementary Fig. S4). These data suggest that TCS, by stimulating the secretion of factors by stromal cells, might promote prostate tumorigenesis (Fig. 6C). **The last paragraph has been deleted.**

DISCUSSION

The present study reports for the first time the potential impact of TCS, a widely used antibacterial in cosmetics and hygienic products, on human prostate cancer associated stromal cells. Indeed, we have shown that TCS induced an important calcium entry in primary cultured stromal cells of human prostate cancer tissues by activating the TRPA1 channel.

The TRPA1 channel has been studied for its role in the (patho)physiology of several organs and its potential as a drug target for the management of various pathological conditions has previously been suggested (38, 39). Initially reported to detect noxious cold (40), TRPA1 receptors have subsequently been shown to act as sensors of many environmental irritants, including acrolein, formaldehyde, zinc, pungent plant ingredients such as mustard oil, cinnamaldehyde, and allicin, as well as endogenously produced substances such as hydrogen peroxide and 4-hydroxynonenal (38). Our data suggest that the antimicrobial agent TCS has to be included in the long list of environmental factors inducing TRPA1 channel activation. However, the fixation site of TCS on TRPA1 channel needs further investigations, using channel mutants in different intracellular and extracellular ligand binding domains of the channel.

We have also shown here for the first time the exclusive expression of TRPA1 channels in human PrSC. According to the published data, the stroma is transformed into “reactive stroma” during early prostate cancer development and it co-evolves with the cancer progression. Previous studies have established that reactive stroma biology influences prostate tumorigenesis and progression. In the present work, we show that TCS, *via* activation of TRPA1, induces VEGF secretion. The VEGF receptors have been shown to be present in epithelial, stromal and endothelial cells (41), suggesting a complex interplay between the cell components of the prostate cancer micro-environment. VEGF can potentiate different functions in prostate cancer, many of which may be responsible for its growth and survival in the androgen-refractory stage, the aggressive form of the disease. The main function of VEGF is its involvement in lymphangiogenesis where the growth and sprouting of lymphatic endothelium occurs from a pre-existing lymphatic vessel (42). Increased lymphatic vessel formation surrounding a tumor may provide potential routes for tumor cells to migrate to distant organs. As a first line of treatment, androgen-deprivation therapy triggers tumor growth arrest and tumor apoptosis in the primary site, and therefore an increase in the metastatic potential which helps the tumor cells to migrate to a different location and survive. Interestingly, several works have shown the potential VEGF-mediated function that may be

important for the growth and survival of prostate cancer from its early phases to transition to the androgen-refractory stage (41). Thus, TCS-induced VEGF secretion might favour the progression of prostate cancer by modulating the growth and transformation of prostate epithelial cells, angiogenesis and finally metastasis. These aspects need to be investigated in *in vivo* studies to address the impact of TCS on the PCa epithelial-stromal and stromal-endothelial cell interactions. Secondly, the impact of TCS on the cancer cell tumor development (in nude mice) and the potential variations of vessel density in these PCa cell xenografts require further investigations. In this context, our preliminary work showed that the TCS and AITC treatment of nude mice co-injected with human prostate cancer epithelial and stromal cells promoted tumor development. These data suggest that targeting the TRPA1 channel could be an alternative to the actual therapies (AR ablation) leading to relapse in the majority of cases.

In addition to environmental factors, several endogenous TRPA1 activators have also been detected. These may modulate the secretion of paracrine factors by stromal cells. TRPA1 is an attractive candidate, because it can be activated by molecules that are produced during oxidative phosphorylation, such as hydrogen peroxide (H₂O₂), 4-Hydroxynonenal (4-HNE), cyclopentenone prostaglandins (PGJ₂) (43), and Methylglyoxal, formed from triose phosphates during secondary glucose metabolism in hyperglycemic condition (39). Recently, TRPA1 has also been shown to be activated by formaldehyde (44).

Interestingly, clinical data have shown that formaldehyde concentration is elevated (2,8 fold) in the urine of patients with prostate and bladder cancer (45) and in the expired air from tumor-bearing mice and breast cancer patients (46). Formaldehyde is considered to be a cancer development risk factor. A recent work showed that in tissues from breast and lung cancer patients, the formaldehyde concentration was 0.75 mM with the highest concentration of 2.35 mM, concentrations being strong enough to activate TRPA1 thereby inducing the secretion of tumor promoting factors (44). These data suggest that cancer-derived substances (formaldehyde) could have pro-cancer effects by activating TRPA1 in stromal cells leading to the secretion of the paracrine factors themselves promoting evolution of cancers towards more aggressive forms.

Overall our data suggest that targeting TRPA1 channel might be an advantage to limit tumor progression. Highlighting the direct effects of Triclosan on the TRPA1 channel expressed in cancer could allow considering new and/or complementary therapies targeting this channel,

and/or preventive measures in the treatment of prostate cancers which take into account the impact of these environmental factors.

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FIGURE LEGENDS

Figure 1. Effects of TCS and TCC on basal $[Ca^{2+}]_i$ of stromal cells derived from prostate cancer. Direct effects of TCS (A) or its analog TCC (B) at different concentrations on $[Ca^{2+}]_i$ were studied by calcium imaging in a 2 mM $CaCl_2$ medium (2 Ca^{2+}). Insets show the respective chemical structure of each compound. TCS induced two types of calcium response depending on the composition of stromal cell population: transient with a subsequent low amplitude plateau phase (C) and transient with a subsequent high amplitude plateau phase (D). Insets in C and D present the expression of the α -actin and vimentin in two different primary cultures of PrSC. To determine the origin of the TCS-induced calcium response, cells were first placed in a Ca^{2+} -free bath solution (0 Ca^{2+}) and then exposed to 2 mM extracellular Ca^{2+} (2 Ca^{2+}) as indicated (E). Effect of TCS on $[Ca^{2+}]_i$ in human Primary Epithelial Cells (hPEC) (F). The TCS application and presence or absence of extracellular Ca^{2+} are marked by horizontal bars. Each experiment was repeated at least 5 times in triplicate on different cell cultures on a field of 25 to 40 cells and representative figures are presented for each experiment.

Figure 2. Suppression of TCS-induced calcium entry by inhibition of TRPA1 in PrSC. Calcium imaging experiment in presence of TCS (A) or Flufenamic acid (B) combined with the non specific TRPA1 inhibitor, Ruthenium Red (RR). (C) TCS-induced calcium increase in presence of the specific inhibitor HC-030031. (D) RT-PCR and western blot performed on PrSC transfected with control siRNA (siCTL) or siTRPA1-1 (25 nM). The corresponding calcium imaging experiments in siCTL and siTRPA1-transfected cells is shown in (E). The functionality of TRPA1 was studied by the application of a known TRPA1 agonist (AITC) in the absence or presence of TRPA1 inhibitor (HC-030031, 200 μ M) (F). Each experiment was repeated at least 3 times in triplicate on different cell cultures on a field of 25 to 40 cells and representative figures are presented for each experiment.

Figure 3. Electrophysiological studies of the TCS-induced TRPA1 activation in PrSC. TCS (10 μ M) increased whole-cell currents at all membrane potentials ranging from -100 to 120 mV in PrSC (A). (B) Representative time courses of whole-cell currents in PrSC measured at membrane potentials of -150 mV (red circles) and +150 mV (black circles) with punctual administrations of HC-030031 (50 μ M). (C, D) Representatives time courses and current-voltage (I-V) relationships of whole-cell currents in PrSC showing that TCS (10 μ M)-induced currents (b) was suppressed by Ruthenium Red (RR, 50 μ M) (c). (E, F)

Representative time courses (a) and current–voltage (I–V) relationships (b) of whole-cell currents in PrSC showing that TCS (10 μ M)-induced currents were similar to AITC (30 μ M)-induced currents which were also inhibited by HC-030031 (HC, 50 μ M).

Figure 4. TCS-induced ion currents in HEK-hTRPA1 cells. Electrophysiological studies were carried out in HEK293 cells transfected either with vector alone (HEK-vector) or with human TRPA1 coding sequences (HEK-hTRPA1). Representative time courses measured at membrane potential of -80 mV (red circles) and +80 mV (A) and current–voltage (I–V) relationships (B) of whole-cell currents recorded in HEK-hTRPA1 cells. The application of TCS (10 μ M) induced similar currents to that of induced by AITC (30 μ M). Both AITC and TCS-induced currents were inhibited by HC-030031 (HC, 50 μ M). Representative time courses measured at membrane potential of -80 mV (red circles) and +80 mV (C) and current–voltage (I–V) relationships (D) of whole-cell currents recorded in HEK-vector cells. The drug applications are marked by horizontal bars.

Figure 5. TRPA1 channel expression prostate cancer tissues. The TRPA1 channel expression was assessed by RT-PCR (A) and Western blotting (B, C) in stromal cells derived from human prostate cancer (PrSC, numbers indicate PrSC from different patients), prostate epithelial cells (LNCaP, PC-3 and DU145 prostate cancer cell lines and hPEC primary epithelial cells), HEK-vector and HEK-hTRPA1 cells. Semi-quantitative RT-PCR experiments were performed to study the expression of TRPA1 (510 bp) mRNA using specific primers. To detect TRPA1 protein, 20 μ g total proteins were analysed by western blot using a TRPA1-specific antibody. Immunofluorescence studies using a TRPA1 antibody (green) were performed on grade 3 PCa FFPE tissues (D, E), on primary stromal cells PrSC (F) and on normal prostate and BPH FFPE tissues (G, H). Nuclei were either stained by DAPI (blue) or propidium iodide (red). The panel (I) shows the control experiment where the primary antibody was omitted and only the nuclei were stained by propidium iodide (PI). Immunofluorescence studies performed on normal breast (J) and breast cancer tissues (K, L) using the TRPA1 antibody (green) and DAPI staining for the nuclei (blue).

Figure 6: TCS induces mitogenic factor secretion. (A) 3×10^3 PC-3 cells grown in 96 well plate in complete medium were either treated with 0.1% FCS medium containing or not TCS (0.1 or 1 μ M) or with media conditioned by PrSC cells exposed or not to TCS (0.1 or 1 μ M) for 3 days. MTS assay was then performed and mean data of viable cells are represented. *,

$P < 0.001$ relative to CTL conditions. **(B)** Quantitative measurement of human VEGF by ELISA in the conditioned media of PrSC cells. Stromal PCa cells were cultured for 4 days in presence or not of TCS (1, 5 or 10 μM) and HC-030031 (200 μM). Experiments were performed as described in *materials and methods* and results are shown as the mean \pm SE. Experiments were repeated four times in independent primary cell cultures and a representative figure is presented. Results are presented as the percentage of control (CTL).

*, $P < 0.01$ relative to CTL condition; ** $P < 0.001$ TCS 5 μM *versus* TCS 5 μM + HC-030031 200 μM . **(C)** Hypothetic TCS-activated pathways in PrSC: TCS, by modifying calcium signalling through TRPA1, could trigger stromal cells secretion of mitogenic factors, leading to the proliferation and/or the migration of PCa epithelial and vascular endothelial cells (angiogenesis), two important components of tumor formation.

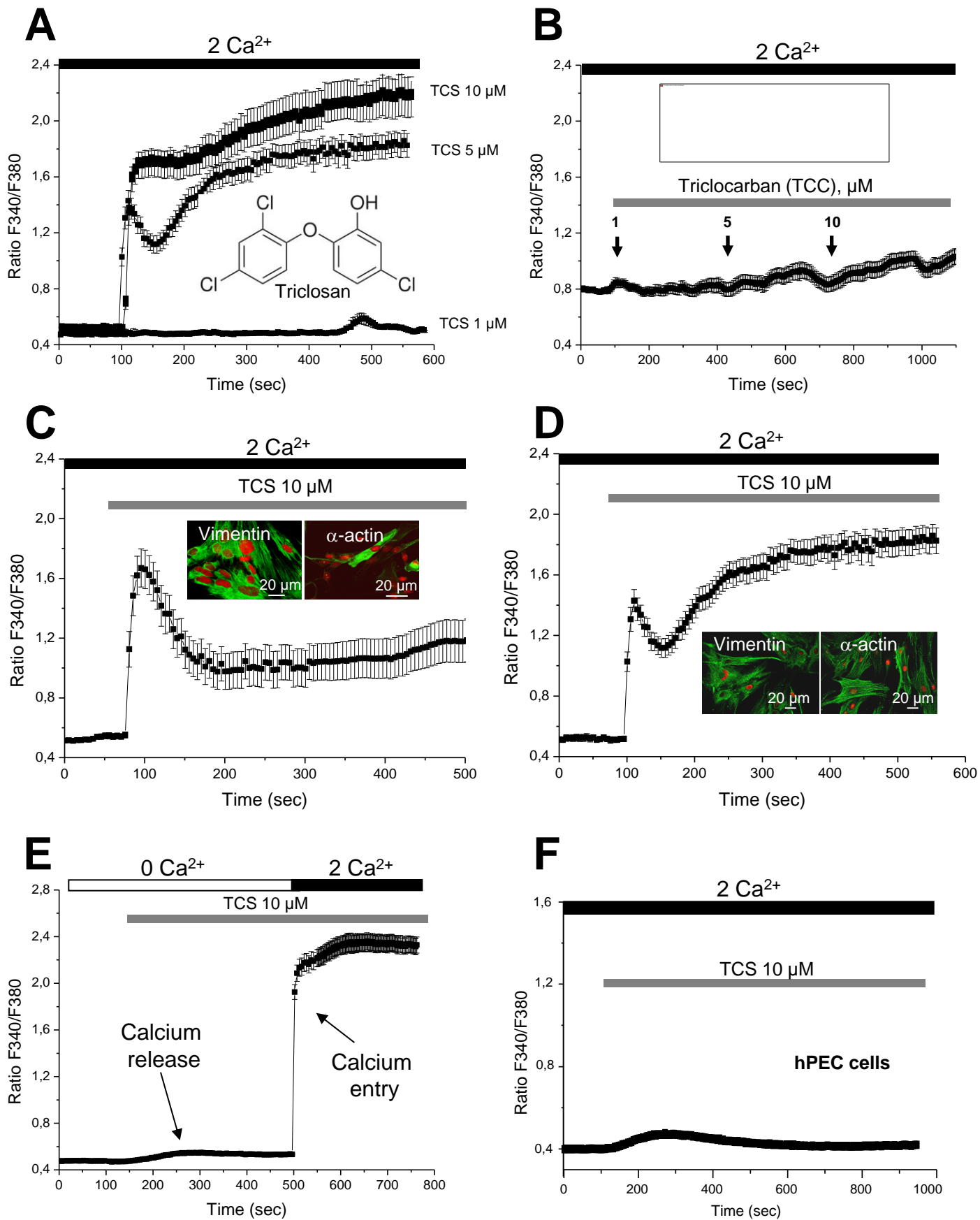


Figure 1: Derouiche et al., 2016

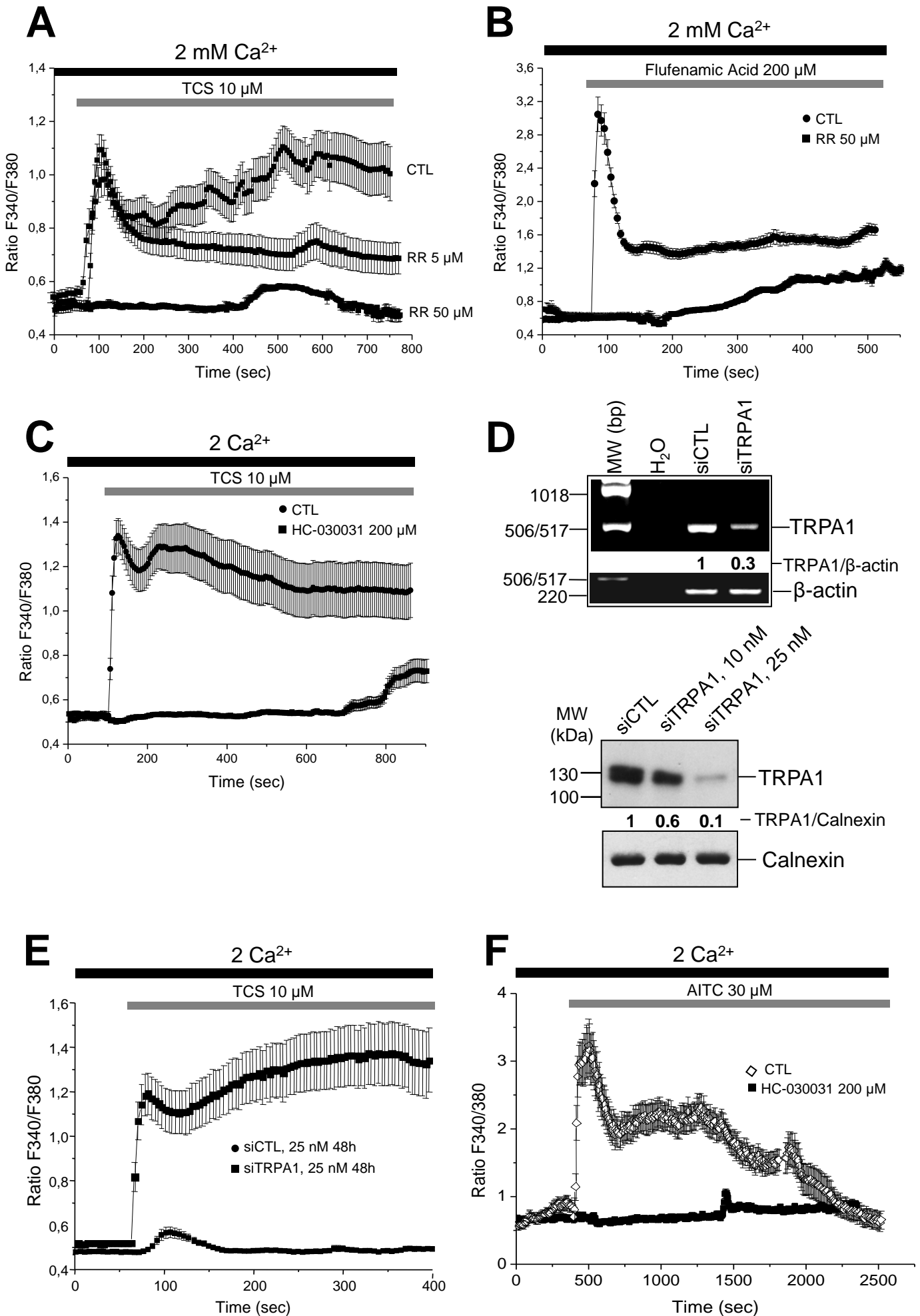


Figure 2: Derouiche et al., 2016

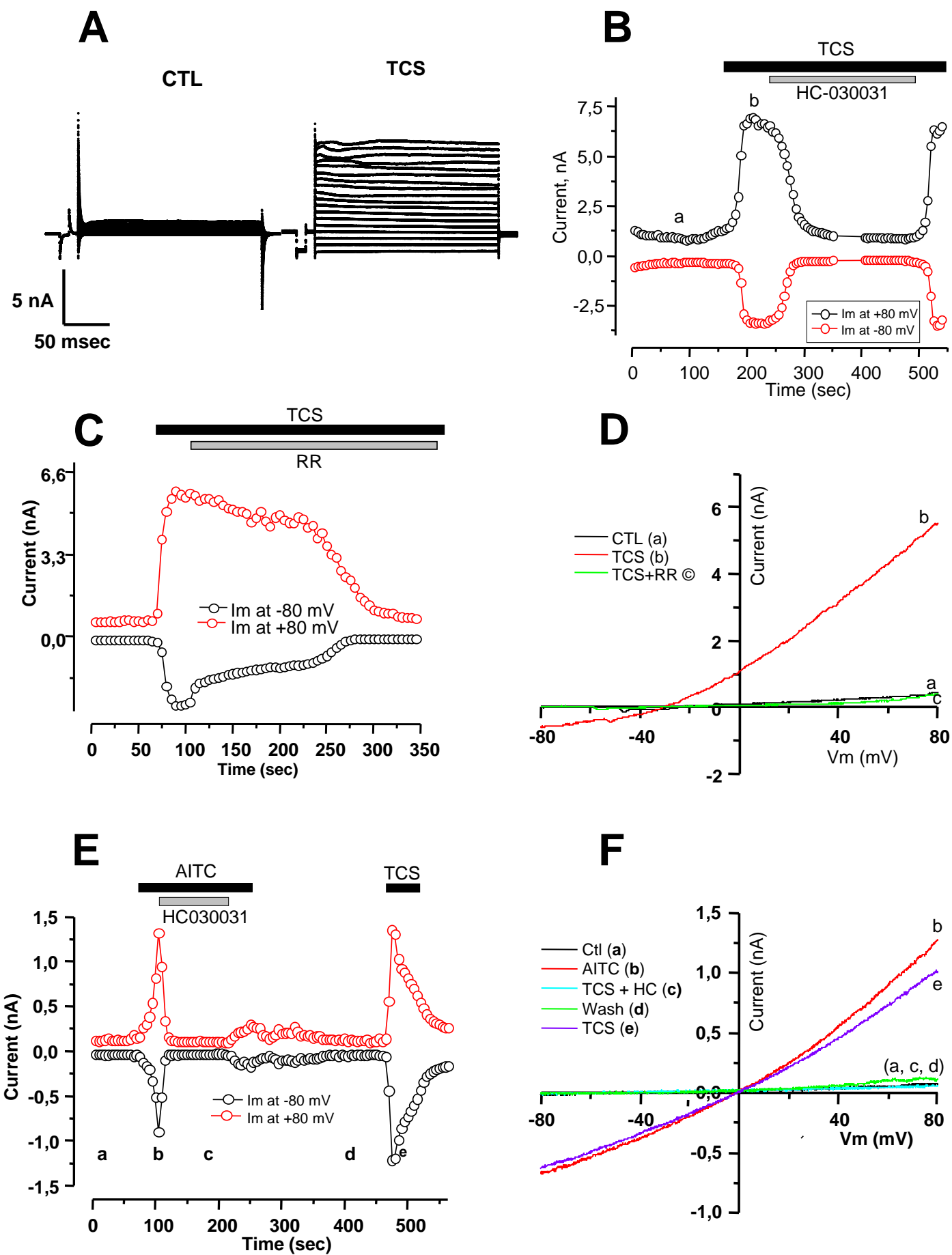


Figure 3: Derouiche et al., 2016

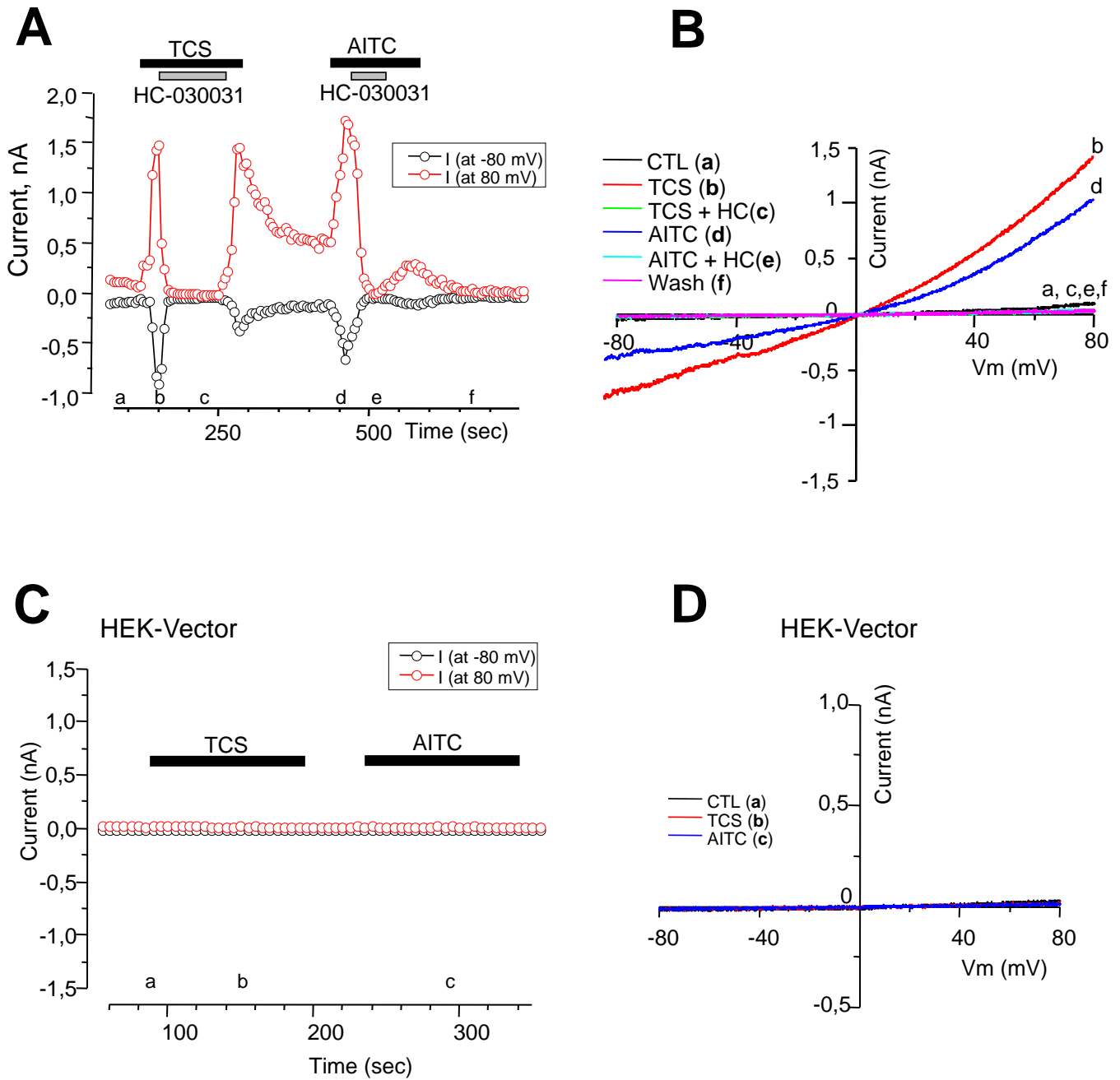


Figure 4: Derouiche et al., 2016

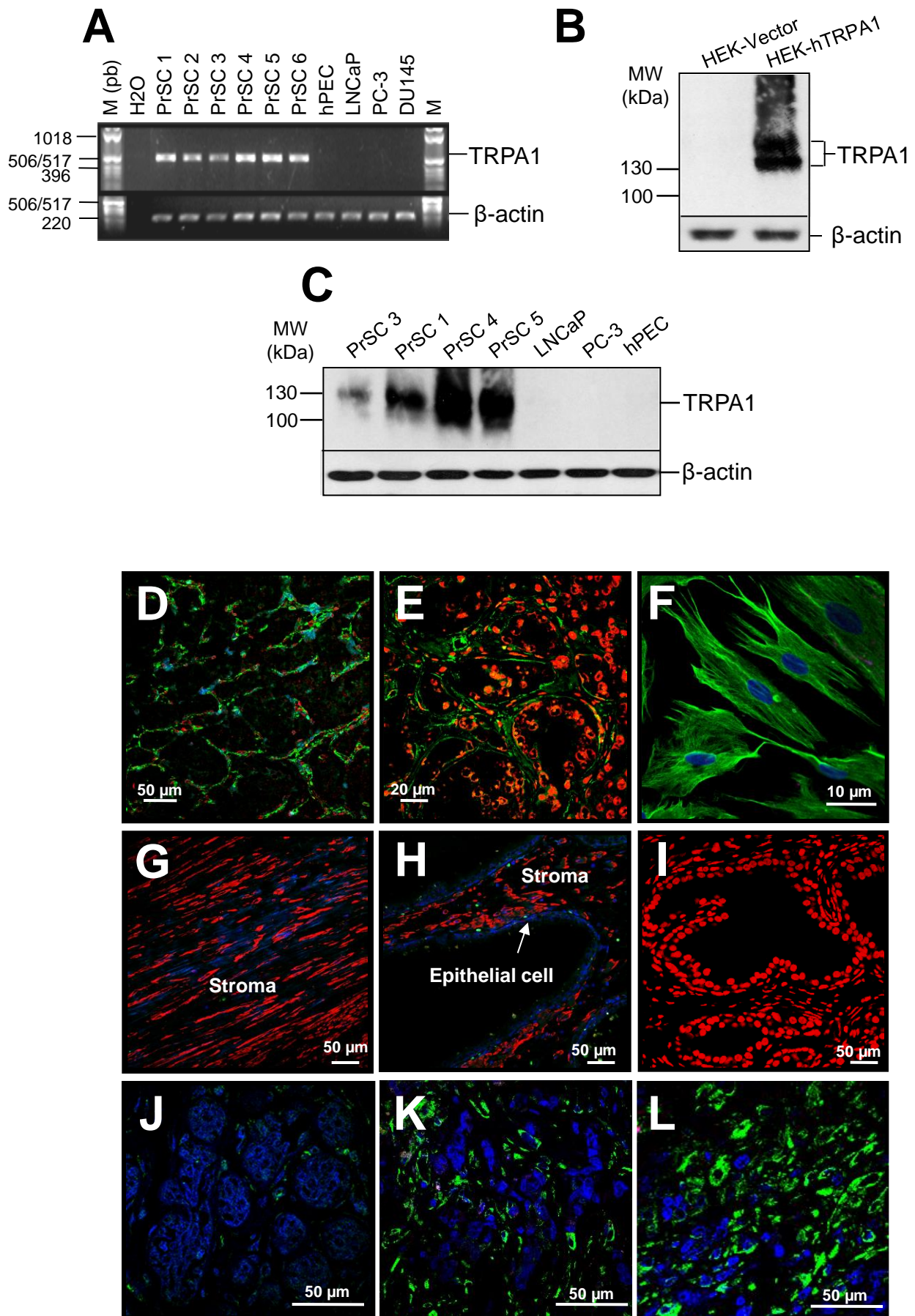


Figure 5: Derouiche et al., 2016

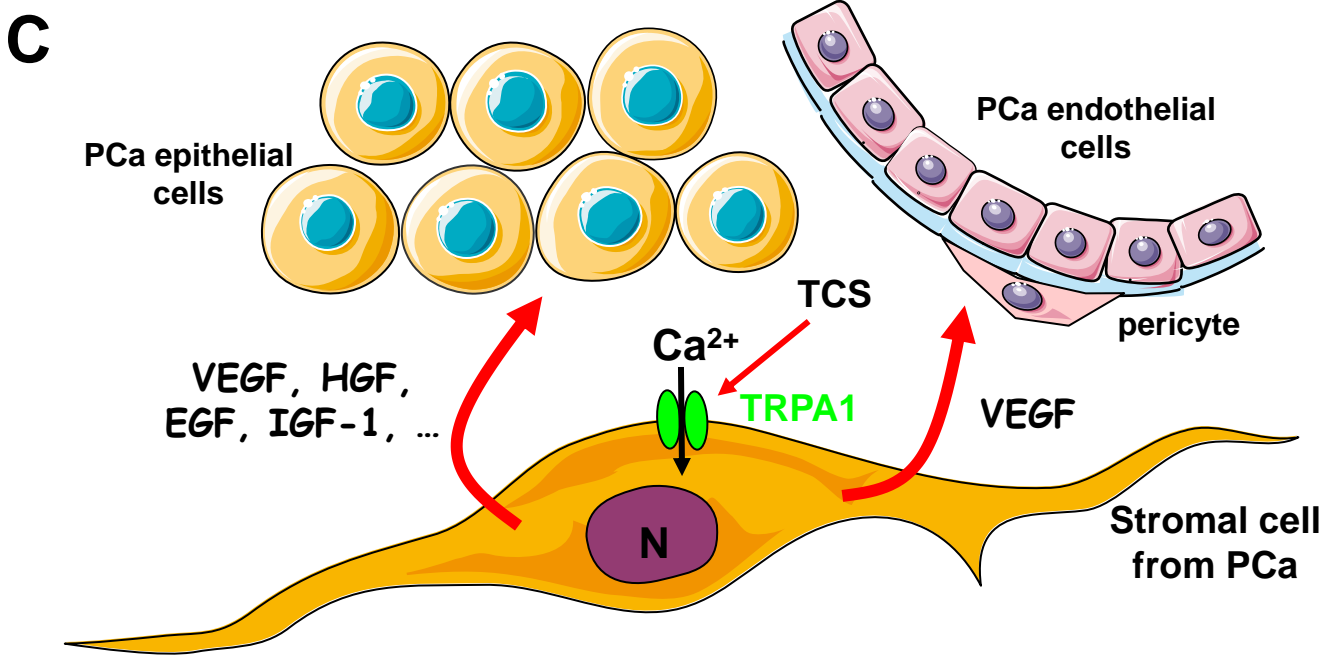
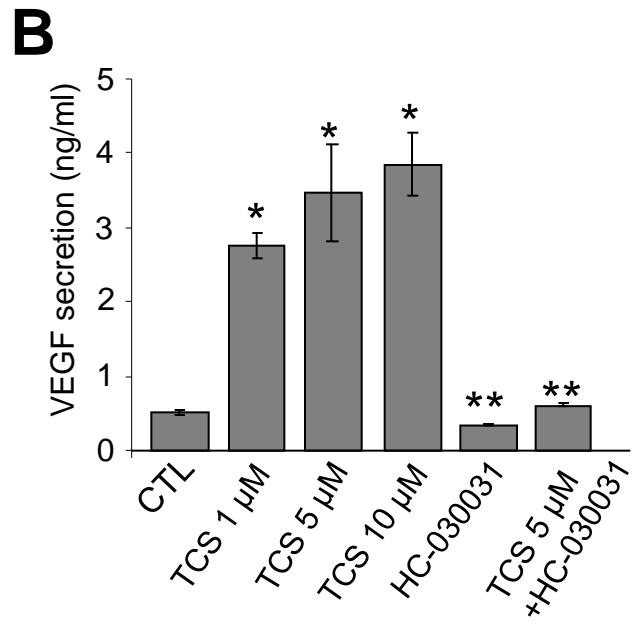
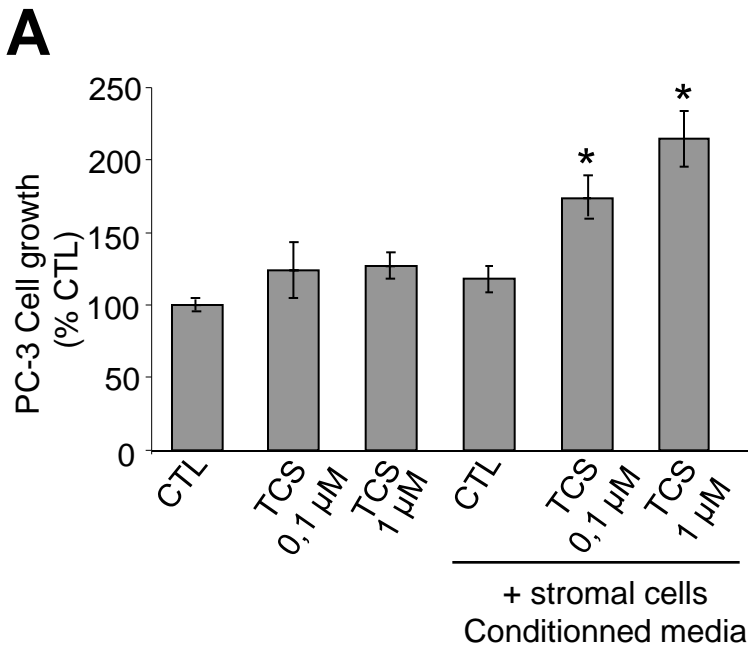


Figure 6: Derouiche et al., 2016