

## Original Article

# Effects of TRPM8 on the proliferation and motility of prostate cancer PC-3 cells

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### Abstract

We investigated the effects of transient receptor potential M8 (TRPM8) channel on the proliferation and motility of androgen-independent prostate cancer PC-3 cells. After being permanently transfected with an empty vector and cDNA encoding the TRPM8 protein, cells were analysed for cell cycle distribution and motility using flow cytometry and scratch assay. Immunocytochemistry and Ca<sup>2+</sup> imaging analysis revealed the overexpression of functional TRPM8 channel on both endoplasmic reticulum and plasma membrane of PC-3-TRPM8 cells. Cell cycle distribution and scratch assay analysis revealed that TRPM8 induced cell cycle arrest at the G<sub>0</sub>/G<sub>1</sub> stage ( $P < 0.05$ ) and facilitated the cell apoptosis induced by starvation ( $P < 0.05$ ). Furthermore, TRPM8 inhibited the migration of PC-3-TRPM8 cells ( $P < 0.01$ ) through the inactivation of focal-adhesion kinase. It appears that TRPM8 was not essential for the survival of PC-3 cells; however, the overexpression of TRPM8 had negative effects on the proliferation and migration of PC-3 cells. Thus, TRPM8 and its agonists may serve as important targets for the treatment of prostate cancer.

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**Keywords:** migration, proliferation, prostate cancer, transient receptor potential (TRP) channels

## 1 Introduction

Prostate cancer (PC) is one of the leading threats to men's health [1]. In its early stages, PC cells depend on androgens for growth and survival, and androgen ablation therapy at that time may be effective in causing tumours to regress; however, in the late androgen-independent stage, there is currently no successful therapy.

The important role of Ca<sup>2+</sup> in global cancer-related cell signalling pathways is well accepted. Fluctuations in Ca<sup>2+</sup> homeostasis may lead to an increase in cell proliferation [2, 3], and even induce differentiation [4] and apoptosis [5–7]. According to a growing number of studies, cationic channels from the transient receptor potential (TRP) family are key players in calcium homeostasis and cell physiopathology. The *Trpm8* gene, initially known as *trp-p8*, encodes for a so-called 'cold' receptor protein belonging to the melastatin (TRPM) subfamily of TRP channels, which is activated by cool temperatures and by menthol. Aside from sensory neurons, in which the role of TRPM8 involves mediating cold-evoked excitation [8], this channel is most abundantly expressed in the prostate tissue. In fact, TRPM8 was first cloned from the human prostate

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as a prostate-specific gene [9], even before its role in mediating the cold sensation response was established. Moreover, while remaining at moderate levels in a normal prostate, TRPM8 expression strongly increases in PC cells, and the expression levels have been shown to be highly correlated with specific PC tumour stages [9].

It has been shown that anti-androgen therapy greatly reduced the expression of TRPM8, suggesting that TRPM8 is regulated by androgens [10]. Bidaux *et al.* [11] also showed that in PC cell lines, and in primary cultures of normal, hyperplastic and cancerous prostate epithelial cells, TRPM8 was a target gene of the androgen receptor (AR). TRPM8 expression-silencing experiments using small interference RNA (siRNA) suggested that  $\text{Ca}^{2+}$  influx through the TRPM8 channel plays an essential role in cellular  $\text{Ca}^{2+}$  homeostasis in prostate epithelial cells and is involved in cell survival [12].

However, upon administration of anti-androgen therapy, the prostate epithelial cells downregulate the expression of AR and, consequently, that of TRPM8 mRNA. Prostate cancer and metastasis then progress into an androgen-independent stage, resulting in cancer relapse with a more aggressive phenotype rather than apoptosis or a simple regress.

This study was designed to investigate the possible effects of TRPM8 on the proliferation and motility of androgen-independent cancer PC-3 cells that are characterized by a decreased or complete lack of expression of AR and TRPM8.

## 2 Materials and methods

### 2.1 Cell culture

Human prostate carcinoma PC-3 cells were purchased from the American Type Culture Collection (ATCC, Manassas, VA, USA). PC-3 cells were routinely cultured in RPMI 1640 medium (Gibco BRL, Grand Island, NY, USA) supplemented with  $100 \text{ IU mL}^{-1}$  penicillin G sodium,  $100 \mu\text{g mL}^{-1}$  streptomycin sulphate and 10% foetal bovine serum (FBS) (Gibco BRL, Grand Island, NY, USA) in a humidified atmosphere consisting of 95% air and 5%  $\text{CO}_2$  at  $37^\circ\text{C}$ .

### 2.2 Permanent transfection of PC-3 cells with TRPM8 cDNA

PC-3 cells were plated on a six-well plate and transfected at about 90% confluence with the rat TRPM8

encoding vector pcDNA3 (a kind gift from Dr David Julius of the University of California, San Francisco, CA, USA) using  $10 \mu\text{L}$  Lipofectamine 2000 (Invitrogen, Carlsbad, CA, USA) as recommended by the manufacturer's protocol. Stably transfected clones were selected using Geneticin G-418 (Sigma, St. Louis, MO, USA) at a concentration of  $500 \mu\text{g mL}^{-1}$ . Nine colonies were identified using reverse transcription-polymerase chain reaction (RT-PCR) and western blot analyses, and were then subcloned and maintained under the selected pressure for an additional week.

### 2.3 Immunofluorescence and microscopy

The PC-3-vector (permanently transfected with plasmid encoding an empty vector) and PC-3-TRPM8 cells (permanently transfected with plasmid encoding the TRPM8 complementary DNA) were plated on 12-mm coverslips and incubated overnight before being fixed with 100% methanol at  $-20^\circ\text{C}$  for 10 min. After three washes in phosphate buffered solution (PBS), the cells were incubated in 5% bovine serum albumin (BSA) at room temperature for 30 min and then washed in PBS thrice. Afterwards, the cells were incubated with a primary rabbit polyclonal anti-TRPM8 antibody (code: ACC-049, Alomone Labs, Jerusalem, Israel) and diluted (1:100) in 1% BSA at room temperature for 1 h. After thorough washes in PBS, the FITC-anti-rabbit secondary antibody (Jackson ImmunoResearch Laboratories, Inc., West Grove, PA, USA) was added to the mixture and incubated for 1 h, and the cells were subsequently washed four times with PBS, followed by incubation with 4,6-diaminodino-2-phenylindole (Sigma). Coverslips were analysed using a confocal microscope.

### 2.4 $\text{Ca}^{2+}$ imaging

The cytosolic  $\text{Ca}^{2+}$  concentration ( $[\text{Ca}^{2+}]_c$ ) was measured using the ratiometric dye Fura-3 (Molecular Probes, Leiden, The Netherlands). The temperature was maintained at  $37^\circ\text{C}$  using a temperature controller (Cell Microcontrol System, Norfolk, VA, USA). For confocal  $\text{Ca}^{2+}$  imaging, the cells were loaded in the presence of  $2.5 \mu\text{mol L}^{-1}$  Fluo-3AM dye in the culture medium at  $37^\circ\text{C}$  for 2 h. During measurements, the cells were incubated in Hanks' balanced salt solution (HBSS) containing  $150 \text{ mmol L}^{-1}$  NaCl,  $5.4 \text{ mmol L}^{-1}$  KCl,  $2 \text{ mmol L}^{-1}$   $\text{CaCl}_2$ ,  $1 \text{ mmol L}^{-1}$   $\text{MgCl}_2$ , and  $10 \text{ mmol L}^{-1}$  Hepes (pH adjusted to 7.4 with  $1 \text{ mol L}^{-1}$  NaOH). In order to produce  $\text{Ca}^{2+}$ -free conditions,  $\text{CaCl}_2$  was removed from this

solution, and 0.5 mmol L<sup>-1</sup> ethylene glycol tetraacetic acid (EGTA) was added in its place. The emission intensity was measured for 90 s at 3-s intervals using excitation wavelengths of 340 and 380 nm at an emission wavelength of 520 nm. Menthol (Sigma) was added after 15 s. Analyses of the emission/excitation intensity ratios at 340 nm/380 nm were performed using the ImageMaster suite of software.

### 2.5 Cell cycle and cell apoptosis induced by starvation were examined by flow cytometry

Approximately  $5 \times 10^5$  cells per well were incubated until 85% confluence was achieved and were then digested with 0.25% trypsin (Gibco BRL, Grand Island, NY, USA). Cells were subsequently harvested and fixed gently (drop by drop) by the addition of 70% ethanol (in PBS) at 4°C overnight and then re-suspended in PBS containing 40 µg mL<sup>-1</sup> propidium iodide (PI), 0.1 mg mL<sup>-1</sup> RNase and 0.1% Triton X-100 in a dark room. After incubation at 37°C for 30 min, the cells were analysed through flow cytometry (Becton-Dickinson, San Jose, CA, USA) equipped with an argon ion laser at a wavelength of 488 nm. The cell cycle stage was then determined and analysed.

For cell apoptosis analysis, cells were seeded in a 75-mm flask at 40%–50% confluence. After 24-h incubation in complete medium, the old medium was aspirated and a culture medium containing 1% FBS was added, after which the cells were incubated for an additional 48 h. After treatment, the cells were incubated in a binding buffer containing FITC-conjugated Annexin V and PI (Abcam, Cambridge, MA, USA) at room temperature for 5 min in the dark, according to the manufacturer's protocol. The percentages of apoptotic cells were then determined using flow cytometry.

### 2.6 Scratch motility assay

Cells were cultured as confluent monolayers in complete medium for 24 h, and then wounded by removing cells across the well with a standard 200 µL pipette tip [13]. The wounded monolayers were washed twice to remove the non-adherent cells. The wound closure was monitored using an inverted phase contrast microscope (Leica, Wetzlar, Germany) at the time the wound was created and 24 h later. This 24-h time interval was chosen as it is shorter than the PC-3 doubling time. Four different fields from each sample were considered for quantitative estimation of the distance between the

borderlines, and in each image, four different equidistant points were measured to obtain a better estimate of the true width of the wounded area. The migration rate is expressed as a percentage of the control (PC-3), and was calculated as the proportion of the mean distance between both borderlines caused by scratching to the distance that remained cell-free after re-growing. Two independent series of experiments were performed in quadruplicates.

### 2.7 Western blot assay

Cultured cells were pooled and lysed in lysis buffer (50 mmol L<sup>-1</sup> Tris-HCl pH 7.4, 5 mmol L<sup>-1</sup> ethylenediaminetetraacetic acid, 1 mmol L<sup>-1</sup> EGTA, 10 mmol L<sup>-1</sup> 2-mercaptoethanol) containing protease inhibitors (5 µg mL<sup>-1</sup> leupeptin, 5 µg mL<sup>-1</sup> aprotinin, 10 µg mL<sup>-1</sup> soybean trypsin inhibitor and 1 mmol L<sup>-1</sup> phenylmethylsulphonyl fluoride) and were subsequently sonicated. After centrifugation at  $12\,000 \times g$  for 15 min to remove all organelles, the supernatant was recovered, and the total protein content was measured using a bicinchoninic acid (BCA) kit. The protein expression of TRPM8, cyclin-dependent kinase (Cdk) 4, Cdk6, focal-adhesion kinase (FAK)-pY397 and glyceraldehyde-3-phosphate dehydrogenase (GAPDH) was assayed using western blot analysis using anti-TRPM8-specific (code: ACC-049, Alomone labs, Jerusalem, Israel), anti-Cdk4-specific (Neomarkers, Union City, CA, USA), anti-Cdk6-specific (Neomarkers), anti-FAK-pY397-specific (Biosource, Camarillo, CA, USA) and anti-GAPDH-specific (Neomarkers) antibodies, as described earlier [7].

### 2.8 Statistical analysis

The SPSS version 11.5 for Windows (SPSS, Chicago, IL, USA) was used for the statistical analysis. All of these data have been presented as the mean  $\pm$  SEM. Statistical analysis was performed using unpaired *t*-test, with *P* < 0.05 taken as statistically significant.

## 3 Results

### 3.1 Detection of TRPM8 protein expression in PC-3, PC-3-vector and PC-3-TRPM8 cells

Using the specific TRPM8 antibody, we investigated the TRPM8 protein expression in PC-3, PC-3-vector and PC-3-TRPM8 cells through analysis of western blot assays (Figure 1A). As expected, we detected the expression of the TRPM8 protein in PC-3-TRPM8 cells

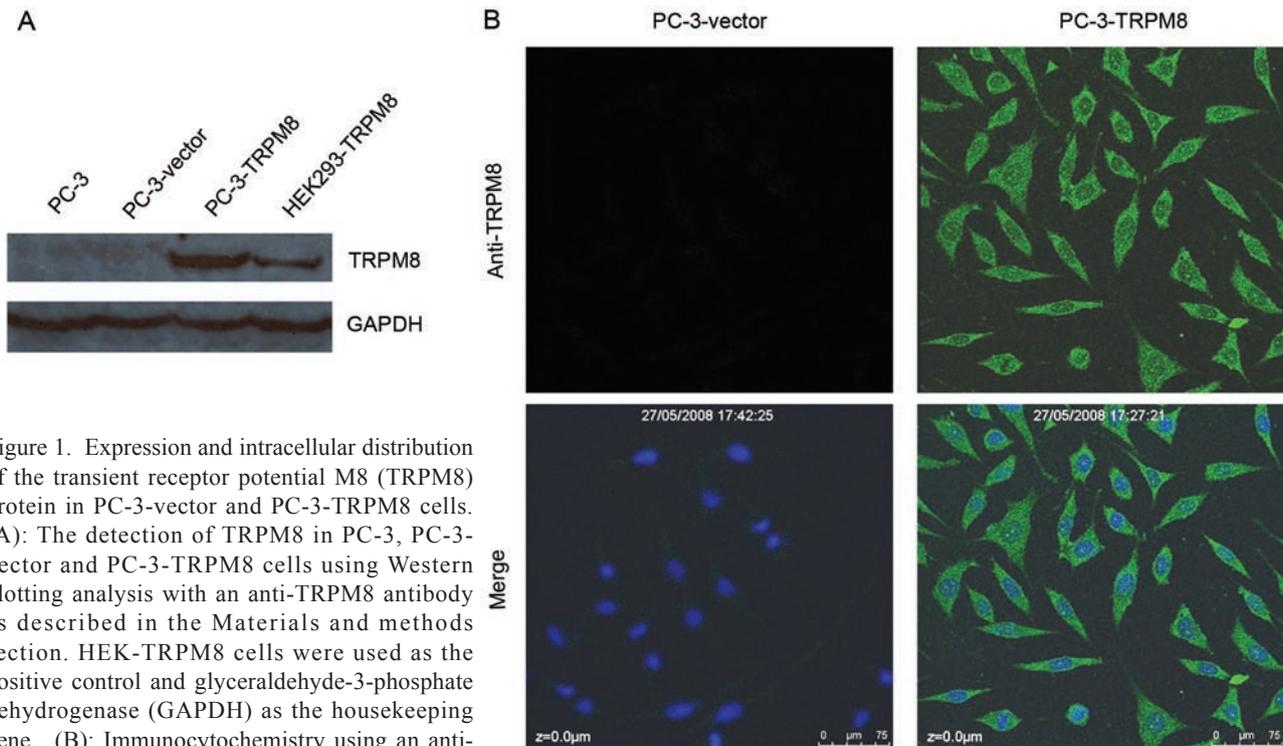


Figure 1. Expression and intracellular distribution of the transient receptor potential M8 (TRPM8) protein in PC-3-vector and PC-3-TRPM8 cells. (A): The detection of TRPM8 in PC-3, PC-3-vector and PC-3-TRPM8 cells using Western blotting analysis with an anti-TRPM8 antibody as described in the Materials and methods section. HEK-TRPM8 cells were used as the positive control and glyceraldehyde-3-phosphate dehydrogenase (GAPDH) as the housekeeping gene. (B): Immunocytochemistry using an anti-TRPM8 antibody as described above. The fluorescence of TRPM8 was determined using confocal microscopy. The results shown are representative of those obtained in each of two independent experiments. Results similar to those shown were obtained for 13 of 15 cells examined in one experiment and for 14 of 15 cells examined in a second experiment.

and in human embryonic KIDNEY-TRPM8 cells, the latter of which served as a positive control. Although it has been reported earlier that TRPM8 is expressed and is functionally active in PC-3 cells [12], we failed to detect marked TRPM8 protein expression. Immunocytochemistry studies using the same antibody showed that TRPM8 immunofluorescence was clearly observed in almost all of the PC-3-TRPM8 cells examined, and that the majority of TRPM8 protein was found to be associated with a reticular structure in the intracellular restricted areas and outside of the nucleus (Figure 1B). Similarly, there was no visible immunofluorescence in the PC-3-vector cells examined.

### 3.2 Increase in $[Ca^{2+}]_c$ in response to menthol in PC-3-vector and PC-3-TRPM8 cells

In a solution containing  $Ca^{2+}$ , both PC-3-vector and PC-3-TRPM8 cells exhibited an increase in the Fluo-3AM fluorescence ratio (representing  $[Ca^{2+}]_c$ ) in response to administration of the TRPM8 agonist menthol ( $100 \mu\text{mol L}^{-1}$ ). However, the increase in  $[Ca^{2+}]_c$  in PC-3-

TRPM8 cells was much higher than that observed for the PC-3-vector cells ( $\Delta\text{Ratio } (340/380)_{\text{mean}}$  of  $2.546 \pm 0.363$  vs.  $0.659 \pm 0.293$ ,  $P < 0.001$ ); notably, these increases appeared as a single peak followed by a low-level sustained plateau (Figure 2).

To test whether the menthol-induced increase in  $[Ca^{2+}]_c$  was the result of the influx of  $Ca^{2+}$  from the extracellular region, cells were incubated in HBSS in the absence of  $Ca^{2+}$  and the effects of menthol were compared with those of cells in a  $Ca^{2+}$ -containing solution. Perhaps of considerably more interest, the increase in Fura-3AM fluorescence in response to the administration of menthol, when compared with those cells in  $Ca^{2+}$ -containing solution, decreased by 32.4% and 39.8% in PC-3-TRPM8 and PC-3-vector cells, respectively (Figure 2).

These results indicate that in both PC-3-vector and PC-3-TRPM8 cells, the substantial increase in  $[Ca^{2+}]_c$  in response to menthol is mainly caused by the release of  $Ca^{2+}$  from intracellular compartments and is only partly due to the influx of extracellular  $Ca^{2+}$ . This means that

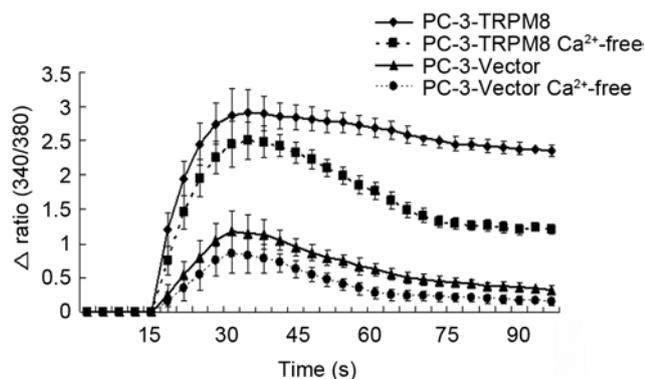


Figure 2. The increase in  $[Ca^{2+}]_c$  in PC-3-vector and PC-3-TRPM8 cells in response to menthol at room temperature (25°C). PC-3-vector and PC-3-TRPM8 cells were treated with 100  $\mu\text{mol L}^{-1}$  menthol in HBBS in the absence and presence of calcium (HBBS- $Ca^{2+}$  and - $Ca^{2+}$ -free, respectively). Each point represents the mean maximal  $[Ca^{2+}]_c$  produced by menthol administration during a 75-s application period. Menthol was added after 15 s.

the TRPM8 protein may be primarily expressed and functionally active on the endoplasmic reticulum (ER) membrane, and only partly expressed on the plasma membrane (PM), although we did not detect its visible expression on the PM when performing the immunofluorescence assay.

### 3.3 Cell cycle and apoptosis were examined using flow cytometry

After cells had been fixed and stained with PI, the DNA content and cell cycle distribution were measured using flow cytometry. The results indicated that the percentage of cells in the  $G_0/G_1$  stage increased for the PC-3-TRPM8 cells when compared with the PC-3 and PC-3-vector cells ( $71.89\% \pm 3.43\%$  vs.  $54.67\% \pm 4.59\%$  and  $71.89\% \pm 3.43\%$  vs.  $51.48\% \pm 3.54\%$ , respectively,  $P < 0.05$ , Figures 3A and C). The effect of the TRPM8 protein on the pro-apoptosis of the PC-3 cells was also investigated. After incubation in RPMI 1640 medium supplemented with 1% FBS in a humidified atmosphere consisting of 95% air and 5%  $CO_2$  at 37°C for 48 h, TRPM8 was found to have a significant pro-apoptotic effect on PC-3 cells, as indicated by the Annexin V-positive ratio in PC-3-TRPM8 cells that was higher than the ratios calculated for PC-3 and PC-3-vector cells ( $12.56\% \pm 1.78\%$  vs.  $4.67\% \pm 1.49\%$  and  $12.56\% \pm 1.78\%$  vs.  $5.28 \pm 1.35\%$ , respectively;  $P < 0.01$ , Figures 3B and D).

### 3.4 Cell migration assay

Using the scratch-wound assay, a continuous and rapid movement was observed for all cells, but a resultant movement of the PC-3 cell migration front was clearly evident at 24 h, in which a highly confluent (90%–100%) monolayer region gradually migrated to the cell-free ‘scratch’ region (Figure 4A). Upon comparison with PC-3 and PC-3-vector cells, the migration of PC-3-TRPM8 cells was significantly reduced after 24 h of incubation ( $P < 0.01$ , Figures 4A and B).

Cell proliferation and migration behaviours, which are both targets of anti-cancer agent development, are important characteristics of cancer cells and serve as indicators of malignance. These observations revealed that the TRPM8 protein resulted in a statistically significant inhibition of PC-3-TRPM8 cell migration as compared with the cell migration observed for either the PC-3 or the PC-3-vector cells (Figure 4B).

### 3.5 Effect of TRPM8 on the expression of proliferation and motility-related proteins

To increase our understanding of the molecular mechanism of TRPM8-induced cell cycle arrest and migration changes in PC-3 cells, we examined the expression of proteins associated with cell cycle and migration after PC-3 cells were transfected with an empty vector and a TRPM8-encoding plasmid. In the group of cell cycle-related proteins, the CDK family is one of the most important and is known for its ability to regulate the cell cycle. However, it is generally thought that FAK plays a key role in cell migration [14], a function required for the invasion and metastasis of cancer cells.

In our study, TRPM8 downregulated the expression of Cdk4 and Cdk6, and thus induced cell cycle arrest at the  $G_0/G_1$  stage (Figure 5). At the same time, TRPM8 was shown to decrease the activation of FAK (Figure 5).

## 4 Discussion

TRPM8 is a so-called ‘cold’ receptor protein belonging to the melastatin (TRPM) subfamily of TRP channels and is activated by both cool temperatures and menthol. Apart from sensory neurons, in which the role of TRPM8 in mediating the cold-evoked excitation has been fairly well established [8, 15], this channel is most abundantly expressed in the prostate. Moreover, although remaining at moderate levels in a normal prostate, TRPM8 expression significantly increases in PC cells. Despite a growing number of studies, the

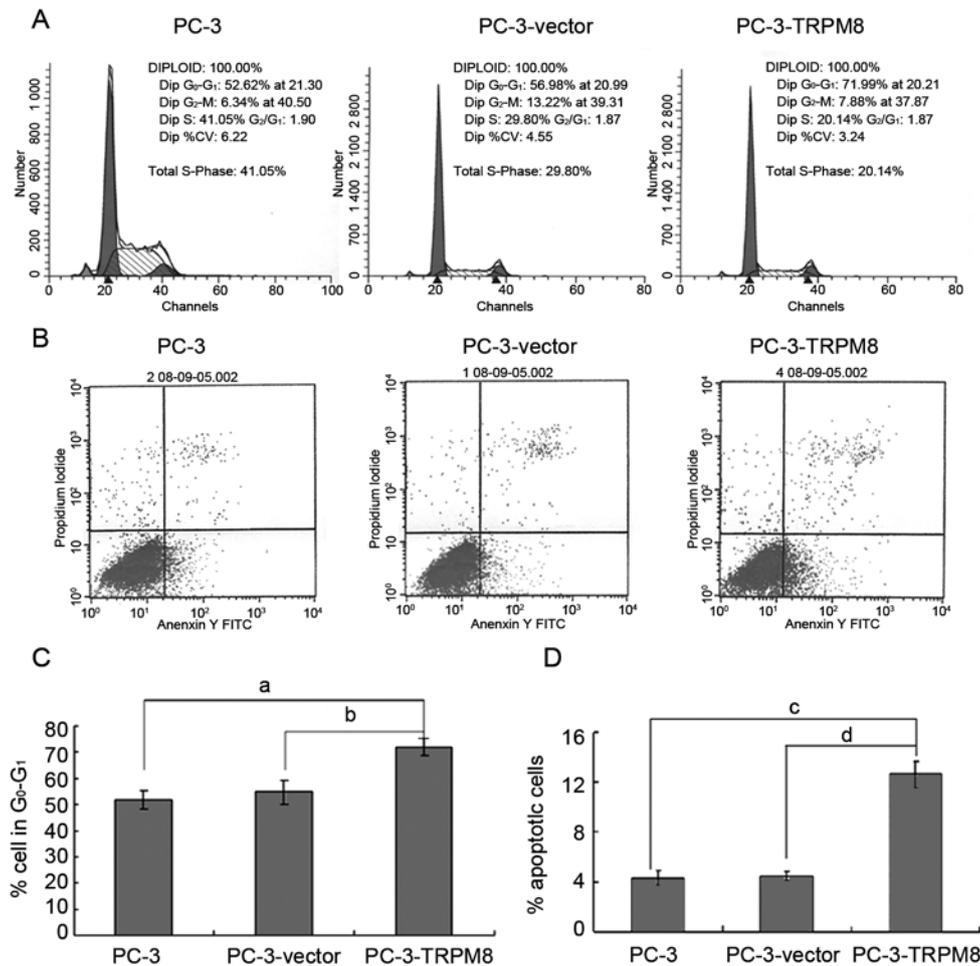


Figure 3. The cell cycle distribution and 1% foetal bovine serum (FBS)-induced apoptosis of PC-3, PC-3-vector and PC-3-transient receptor potential M8 (TRPM8) cells. (A) and (C): Cells stained with PI were analysed to determine the progression through the cell cycle using flow cytometry (<sup>a,b</sup> $P < 0.05$ ,  $n = 3$ , in separate cell cultures). (B) and (D): After treatment with RPMI 1640 supplemented with 1% FBS for 48 h, the cells were incubated with FITC-conjugated Annexin V and PI at room temperature for 5 min in the dark. The percentages of cell apoptosis were then determined using flow cytometry. The data are the mean of three independent experiments performed in duplicate (<sup>c,d</sup> $P < 0.01$ ).

precise role of TRPM8 in the prostate remains unclear, although it has been suggested to be involved in the secretion functions of the prostate, and in the regulation of proliferation and/or apoptosis [11, 12].

Bidaux *et al.* [16] showed earlier that only highly differentiated human prostate primary luminal epithelial cells expressed functional PM TRPM8 channels [<sub>PM</sub>TRPM8 (PM TRPM8)], whereas ER TRPM8 channels [<sub>ER</sub>TRPM8 (ER TRPM8)] remained functional as an ER Ca<sup>2+</sup> release channel independent of its differentiation status. In other words, these results indicate that localization may depend on the epithelial cell phenotype (i.e.,

fully differentiated secretory apical cells versus non-differentiated basal cells) and on the androgen status (i.e., androgen-dependent vs. hormone refractory cells highly resistant to apoptosis).

Although we were unable to detect marked expression of TRPM8 in PC-3-vector cells through immunocytochemistry and western blotting studies, the present investigation confirms that PC-3 cells may express an extremely low level of functional TRPM8, as the specific agonist menthol was able to induce an increase in [Ca<sup>2+</sup>]<sub>c</sub>, both in the presence and absence of Ca<sup>2+</sup>. Although the reasons for the absence of TRPM8

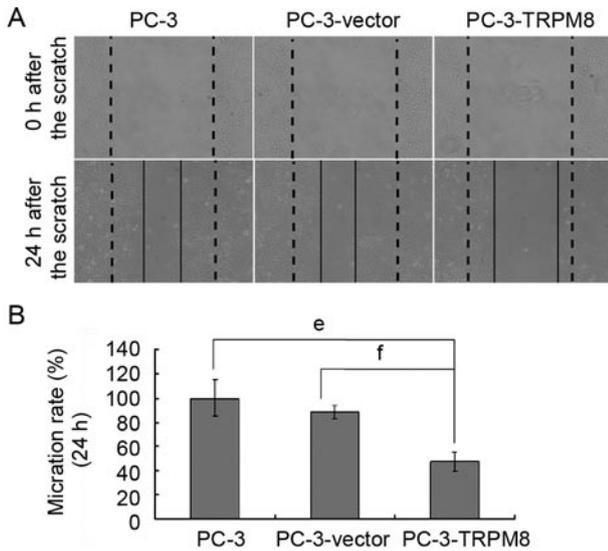


Figure 4. Transient receptor potential M8 (TRPM8) reduces PC-3 cell motility. (A): Photomicrographs showing the representative views of motility assays, obtained at 0 and 24 h. The broad dashed lines represent the original borderlines at the time of scratching, whereas the thin and solid lines represent the borderlines 24 h after scratching (original magnification:  $\times 200$ ). In the control (PC-3 cells) and in the PC-3-vector cells, the distance between the borderlines becomes significantly shorter 24 h after generation of the wound, whereas it is still elevated in PC-3-TRPM8 samples. (B): Wound areas were measured and normalized relative to the control values (PC-3 at 24 h) that were assumed at 100%. The data from six independent experiments were collected and the mean values were plotted with SEM. The graph shows that the cell migration rates of the PC-3-TRPM8 cells are significantly reduced in comparison with the PC-3 and PC-3-vector cells ( $^c, ^fP < 0.01$ ).

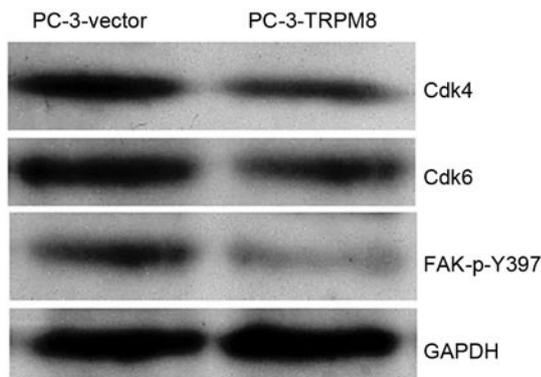


Figure 5. Changes in the protein expression of Cdk4, Cdk6 and FAK-p-Y397. Immunoblot analysis of the protein expression of Cdk4, Cdk6, FAK-p-Y397 and glyceraldehyde-3-phosphate dehydrogenase (GAPDH). The protein expression of GAPDH in the same samples was used as a loading control.

detection in the current study are unclear, this may be related to the antibody itself, in terms of its sensitivity, for example, or to the specific cell line used herein, as we did not detect the expression of TRPM8 in PC-3 cells at the mRNA level through routine RT-PCR. Furthermore, the increase in  $[Ca^{2+}]_c$  induced by menthol in the  $Ca^{2+}$ -containing solution was significantly higher than that observed in the  $Ca^{2+}$ -free solution. The extremely low level of AR expression in androgen refractory prostate cancer PC-3 cells probably contributed to the much lower level of TRPM8 gene expression and the lack of response to androgen regulation [12].

Our results, coupled with those reported by Zhang and Barritt [12], indicate that TRPM8 may be expressed not just from the PM, but also from the ER membrane. Dual localization and the channel-like function of the TRPM8 protein in the two membranes significantly broaden the spectrum of physiological and pathological processes in which it may be involved.

Antagonist experiments using capsazepine and TRPM8 expression-silencing experiments using siRNA [11, 12] suggested that the  $Ca^{2+}$  influx mediated by TRPM8 plays an essential role in cellular  $Ca^{2+}$  homeostasis in LNCaP cells and is involved in cell survival. These results indicate that TRPM8 is an important determiner of  $Ca^{2+}$  homeostasis in prostate epithelial cells, and may be a potential drug target for the management of PC. However, in this study, we found that the increased expression of TRPM8 in androgen-independent PC-3 cells induced cell cycle arrest at the  $G_0/G_1$  stage, as opposed to functioning as an essential component for cell survival as reported earlier [11, 12], or for promoting the proliferation of PC-3 cells. Similarly, a report by Altuwajri *et al.* [17] has shown that dihydrotestosterone at a concentration of 0.001–10  $nmol L^{-1}$  induces cell cycle arrest or inhibits proliferation of PC-3-AR cells, which overexpress heterologous AR. In addition, it has been shown that heterologous AR overexpression in PC-3 cells strongly upregulates the expression of functional TRPM8 at both the levels of transcription and translation [16].

Cell cycle checkpoints are important control mechanisms that ensure the proper execution of cell cycle events. In addition, cell cycle progression is precisely regulated by a series of CDKs whose activities are strongly dependent on their association with cyclin subunits [18]. We found herein that the overexpression of TRPM8 arrested the cell cycle at the  $G_1$  to S phase transition through the downregulation of Cdk4 and Cdk6.

We also found that the overexpression of TRPM8 did not induce cell apoptosis (data not shown), but rather facilitated cell apoptosis induced by starvation in PC-3 cells. These results indicate that TRPM8, although expressed at an extremely low level on both the PM and the ER, may play an important role in maintaining the cytosolic  $\text{Ca}^{2+}$  homeostasis in PC-3 cells. However, once the expression level is significantly increased, TRPM8 may function to perturb the  $\text{Ca}^{2+}$  homeostasis in PC-3 cells, and thus induce an anti-proliferation and pro-apoptotic effect in PC-3 cells.

Death from cancer is generally associated with metastasis, a biological phenomenon linked to the tumour cell's ability to migrate, seed and colonize distant sites. FAK is an important regulator of cell migration [14], and is absolutely required from a functional perspective for the invasion and metastasis of cancer cells. This migration requires individual cells, or more likely small groups of cells, to initially move three-dimensionally through the extracellular matrix (ECM) surrounding the region of the primary tumour. This often, but not always, requires the combined action of matrix metalloproteinases that function to degrade ECM barriers. However, recent evidence suggests that there are also proteolysis-independent invasion pathways, and thus it is possible that FAK may be differentially required for distinct modes of tumour cell migration and invasion. The present investigation indicated that overexpression of TRPM8, through inactivation of FAK, reduced the motility of PC-3 cells.

Recent work by Zhang and Barritt [12] suggested that TRPM8 was required for the survival of the androgen-dependent LNCaP cell line, which strongly expresses TRPM8 on both the PM and the ER under the control of androgen. However, we found that as an androgen-independent cell line, PC-3 cells may express an extremely low level of functional TRPM8 on both the PM and the ER. Overexpression of TRPM8 in PC-3 cells induced cell cycle arrest and facilitated starvation-induced cell apoptosis, whereby TRPM8 itself did not directly induce apoptosis. Furthermore, the overexpression of TRPM8 in PC-3 cells also decreased the motility of the cell through the inactivation of FAK.

Although the natural TRPM8 activators in the prostate have not yet been identified, it is assumed that this cationic  $\text{Ca}^{2+}$ -permeable ion channel plays a functional role in  $\text{Ca}^{2+}$  homeostasis in prostate cells, especially in androgen-dependent cells such as normal prostate cells and LNCaP. We also assume that in PC-3 cells, which

express an extremely low level of TRPM8, this cationic  $\text{Ca}^{2+}$ -permeable ion channel is also of importance and its overexpression may perturb the  $\text{Ca}^{2+}$  homeostasis. Thus, TRPM8 has an anti-proliferation effect on PC-3 cells, and furthermore, TRPM8 has an inhibitive effect on malignant progression through the inactivation of FAK, a protein that mediates both tumour formation and malignant progression.

In summary, this study shows that PC-3 cells express an extremely low level of TRPM8 on both the PM and the ER. Our results have also indicated that overexpression of TRPM8 has negative consequences on the proliferation and malignant progression of PC-3 cells. Therefore, for patients in the late androgen-independent stage, although there is currently no successful therapy, the activation of the existing channels or the overexpression of the channel may serve as a potential alternative treatment that is worthy of further investigation.

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