

# Effects of TRPM8 on the proliferation and angiogenesis of prostate cancer PC-3 cells *in vivo*

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**Abstract.** Prostate cancer is a significant health concern. In the early stages, prostate cancer cells depend on androgens for growth and survival, hence androgen-ablation therapy at this time may be effective in causing tumor regression. However, treatment options for advanced hormone-refractory prostate cancers are still relatively inefficient. This study aimed to investigate the possible effects of TRPM8 on the proliferation and angiogenesis of androgen-independent cancer PC-3 cells *in vivo*. Thirty male nude mice were divided into three groups: the PC-3, PC-3-vector and PC-3-TRPM8 groups. PC-3, PC-3-vector and PC-3-TRPM8 cells were respectively inoculated in the right flank to establish a transplanted tumor model. The mice were treated daily for four weeks and each group was examined by histology and immunohistochemical staining for CD34, FAK and PCNA. A CD34 marked microvascular density (MVD) test was performed. Western blot analysis was used to detect the VEGF protein expression level. Compared to the PC-3 and PC-3-vector groups, the PC-3-TRPM8 group revealed a decrease in tumor volume ( $P=0.000$  and  $P=0.000$ , respectively), MVD ( $P=0.045$  and  $P=0.041$ , respectively), VEGF ( $P=0.000$  and  $P=0.000$ , respectively), FAK and PCNA. The correlation between MVD and VEGF was positive ( $r=0.419$ ;  $P=0.021$ ). These data show that the overexpression of TRPM8 had a negative effect on the proliferation and angiogenesis progression of PC-3 cells *in vivo*.

## Introduction

Prostate cancer (PC) is a major health problem, accounting for a quarter of the new cancer cases diagnosed in adult males in America each year, and accounting for approximately 9% of cancer-related mortality in the same population (1). In the early stages, prostate cancer cells depend on androgens for growth and survival, hence androgen-ablation therapy at this

time may be effective in causing tumor regression. However, treatment options for advanced hormone-refractory prostate cancers (HRPC) are still relatively inefficient (2).

The role of  $Ca^{2+}$  is well established in the majority of the cell signaling pathways involved in carcinogenesis (3). Calcium-permeable channels are potential candidates for involvement in  $Ca^{2+}$  homeostasis in prostate cancer cells. One transient receptor potential (TRP) superfamily of cation channels is of particular interest. The human *trpm8* gene, initially known as *trp-p8*, has been shown to be mainly expressed in the prostate and is overexpressed in prostate cancer (4). The precise physiological function of the TRPM8 channel in normal and cancer prostate tissue remains unknown. TRPM8 expression is markedly upregulated in PC and in other tumors, suggesting a significant role in carcinogenesis (4). It has been shown that anti-androgen therapy greatly reduces the expression of TRPM8, suggesting that TRPM8 is regulated by androgens (5). TRPM8 expression-silencing experiments using small interfering RNA (siRNA) suggested that  $Ca^{2+}$  influx through this channel plays an essential role in cellular  $Ca^{2+}$  homeostasis in prostate epithelial cells and is involved in cell survival (6). Our previous study revealed that PC-3 cells express an extremely low level of TRPM8, and that overexpression of TRPM8 has a negative effect on the proliferation and malignant progression of PC-3 cells *in vitro* (7).

However, upon administration of anti-androgen therapy, the prostate epithelial cells downregulate the expression of androgen receptor (AR) and, consequently, that of TRPM8 mRNA. Prostate cancer and metastasis then progress into an androgen-independent (AI) stage, resulting in cancer relapse with a more aggressive phenotype.

It is well known that angiogenesis is essential for tumor progression and metastasis (8). In relation to PC, it has also been suggested that the degree of tumor angiogenesis is correlated to clinical stage (9). Various endothelial growth factors have been shown to play crucial roles in tumor angiogenesis. Vascular endothelial growth factor (VEGF) is one of the most potent and specific angiogenic factors. Immunohistochemical studies have revealed that PC cells produce VEGF (10,11) and that VEGF expression correlates with microvessel density (MVD) and tumor progression (12).

This study was designed to investigate the possible effects of TRPM8 on the proliferation and angiogenesis of androgen-independent cancer PC-3 cells *in vivo*.

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## Materials and methods

**Cell culture.** PC-3 cells were purchased from the American Type Culture Collection (ATCC, Manassas, VA, USA). PC-3-m8 cells were previously established in our laboratory. Cells were cultured as previously described (7).

**Animals.** Thirty 5-week old male nude mice (weight range 15-18 g) were obtained from the Hubei Provincial Experimental Animal Center, China. All animal study protocols were approved by internationally accepted principles and the Guidelines for the Care and Use of Laboratory Animals of Wuhan University.

**Animal grouping.** The animals were randomized into 3 groups: Group A (PC-3 cell group), group B (PC-3-vector group) and group C (PC-3-TRPM8 group).

**Tumor models.** PC-3, PC-3-vector and PC-3-TRPM8 cells growing exponentially were each implanted into 10 male nude mice by subcutaneous (SC) injection of  $1 \times 10^6$  cells (in 200  $\mu$ l phosphate-buffered saline) into the right flank.

**Observation on growing condition of mice.** The inoculated mice were fed in the Experimental Animal Center of Wuhan University and monitored daily for clinical signs. Tumor measurements were performed every three days and the tumor volume was calculated according to the formula:  $V = (\pi/6)(d_1 \times d_2)^{3/2}$  (13), where  $d_1$  and  $d_2$  are perpendicular tumor diameters.

**Sample collection.** Twenty-eight days after the inoculation of cells, each mouse was injected with 10% chloral hydrate for hyperanesthesia. The mice were sacrificed by decapitation and tumors were removed from the body. One section of the tumor was fixed in formalin for paraffin embedding and one section was snap-frozen in liquid nitrogen and stored at  $-80^\circ\text{C}$ .

**H&E staining assay.** Paraffin-embedded tissues were cut into 4- $\mu$ m slices and deparaffinated in dimethylbenzene for 5-10 min. Then the tissues were put into 100, 95, 85 and 70% alcohol for 2-5 min in turn and finally washed with distilled water and immersed in staining solution. Following hematoxylin staining for 5-15 min, the excess stain solution on the slides was washed off, and color separation with 0.5-1% hydrochloride alcohol was performed for approximately 10 sec. After washing in running water for 15-30 min, the tissues were stained by 0.1-0.5% eosin for 1-5 min. The tissues were then dehydrated with 75, 85, 95 and 100% alcohol for 2-3 min in turn prior to hyalinization with dimethylbenzene twice for approximately 10 min in total. Finally, neutral gum was dropped onto the slip, and then the slip was covered by a slide. As a result, nuclei were stained blue and cytoplasm and collagen fibers were stained various shades of red or pink.

**Immunohistochemical assay.** The streptavidin-peroxidase-biotin (SP) method was used for immunohistochemistry. The slides were deparaffinized conventionally and were immersed in 3%  $\text{H}_2\text{O}_2$  for 10 min to block endogenous peroxidase. Following antigen retrieval by microwave, newborn calf

serum was added for blocking for 10 min. The primary antibody (1:50) was then added for incubation overnight ( $4^\circ\text{C}$ ) and secondary antibodies were added for incubation for 20 min at room temperature. Then streptavidin-biotin-peroxidase solution was used for incubation for 30 min and 3,3'-diaminobenzidine (DAB) was added to the chlorate for 15 min. This was followed by hematoxylin staining, dehydration and hyalinization, and the slip was then covered.

**CD34 marked MVD test.** CD34 is expressed in vascular endothelial cells, tumor cytoplasm or membrane and is used as a specific marker of vascular endothelial cells. By immunohistochemical staining it reveals a distribution of brown or light brown solid bud-like or cord-like blood vessels. Low magnification ( $\times 100$ ) was used to review the microvascular staining in each section and determine the maximum microvascular staining regions, and then the vascular endothelial cells or cell groups which appeared brown at high magnification ( $\times 200$ ) were counted. Each cell group counted as independent micrangium on condition of an obvious distinction from neighboring micrangium and tumorous cell. Five counts of micrangium of each slide at high magnification vision were recorded, and the average was taken as the MVD.

**Detection of VEGF protein expression by Western blotting.** One hundred milligrams of tumor tissue was obtained. The protein lysate was added according to quality/volume (w/v, mg/ $\mu$ l) at a concentration of 1:5, fully homogenized, placed on ice for 30 min and then centrifuged for 5 min at  $260 \times g$ . The total protein content was measured using a bicinchoninic acid (BCA) kit. The protein expression of VEGF and  $\beta$ -actin was assayed using Western blot analysis using anti-VEGF-specific and anti- $\beta$ -actin-specific antibodies (Santa Cruz Biotechnology, Santa Cruz, CA, USA).

**Statistical analysis.** Measurement data were shown as the mean  $\pm$  SD. Groups were compared with the one-way ANOVA analysis. The Spearman coefficient was used to analyse the correlation between MVD and VEGF.  $P < 0.05$  was considered to be statistically significant. All of the data were analyzed with SPSS 17.0.

## Results

**Observation on mice growing conditions and behavior.** In group C, the response to stimuli, activity level, and appetite of each mouse was similar to that in groups A and B, and the body weight did not change significantly (data not shown).

**Observation on tumor growth.** The tumor formation rate in each group was 100%. The tumor growth was infiltrative with a round or oval shape. The tumor volume in group C was less than that of groups A and B, and there was a difference in the volume of the graft between group C and group A or B ( $P = 0.000$  and  $P = 0.000$ , respectively; Fig. 1).

**Histopathologic observation.** Following H&E staining, the transplanted tumors in each group revealed glands of unequal size, which integrated to become lamellar, solid-like or acne-like accompanied with diffuse infiltration when observed

Table I. Comparison of Gleason score in each group (values are presented as the mean  $\pm$  SD).

Group	Gleason score
PC-3	8.7 $\pm$ 0.48
PC-3-vector	8.7 $\pm$ 0.48 <sup>a</sup>
PC-3-TRPM8	8.4 $\pm$ 0.52 <sup>b</sup>

<sup>a</sup>Compared with PC-3 group,  $P>0.05$  ( $P=1$ ); <sup>b</sup>Compared with PC-3 and PC-3-vector group,  $P>0.05$  ( $P=0.186$ ).

Table II. MVD values of tumor in each group (values are presented as the mean  $\pm$  SD).

Group	MVD in tumor
PC-3	38.82 $\pm$ 12.11
PC-3-vector	37.50 $\pm$ 9.97 <sup>a</sup>
PC-3-TRPM8	29.66 $\pm$ 6.04 <sup>b,c</sup>

<sup>a</sup>Compared with PC-3 group,  $P>0.05$  ( $P=0.983$ ); <sup>b</sup>Compared with PC-3 group,  $P<0.05$  ( $P=0.045$ ); <sup>c</sup>Compared with PC-3-vector group,  $P<0.05$  ( $P=0.041$ ).

Table III. Expression of VEGF protein in each group (values are presented as the mean  $\pm$  SD).

Group	VEGF expression in tumor
PC-3	0.885 $\pm$ 0.08
PC-3-vector	0.904 $\pm$ 0.06 <sup>a</sup>
PC-3-TRPM8	0.708 $\pm$ 0.11 <sup>b,c</sup>

<sup>a</sup>Compared with PC-3 group,  $P>0.05$  ( $P=0.614$ ); <sup>b</sup>Compared with PC-3 group,  $P<0.01$  ( $P=0.000$ ); <sup>c</sup>Compared with PC-3-vector group,  $P<0.01$  ( $P=0.000$ ).

under a light microscope. Pathological caryokinesis and low-degree differentiation implied a high degree of malignancy. There was no difference in the Gleason score between the three groups ( $P>0.05$ ; Table I).

**MVD count of the different groups.** To evaluate tumor neovascularization, we immunostained tissue samples using a CD34 antibody (Abcam, Cambridge, UK). CD34 is a cell surface sialomucin widely used as a marker of most vascular endothelial cells, including those of capillaries in the majority of tissues. In the present study, the MVD in groups A and B was found to be higher than that in group C ( $P=0.045$  and  $P=0.041$ , respectively; Fig. 2G, H and I; Table II).

**Expression of VEGF protein in each group.** All groups revealed VEGF protein expression. The expression of VEGF protein in groups A and B was higher than that in group C ( $P=0.000$  and  $P=0.000$ , respectively; Fig. 3).

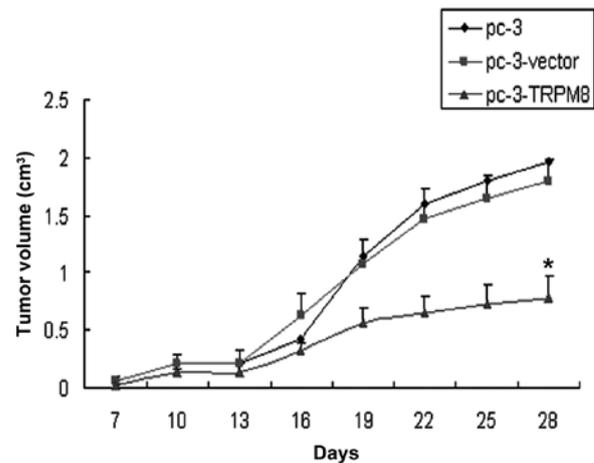


Figure 1. Time course changes of tumor growth in each group. The tumor volume in the PC-3 and PC-3-vector groups was higher than that in the PC-3-TRPM8 group. \* $P<0.05$ .

**Relationship between MVD and VEGF protein expression in each group.** MVD and VEGF expression in tumor tissue had a correlation coefficient of  $r=0.419$  ( $P=0.021$ ) for all three groups.

**Expression of focal adhesion kinase (FAK) and proliferating cell nuclear antigen (PCNA) in each group.** FAK is a non-receptor protein tyrosine kinase that regulates adhesion-dependent cell signaling (14). In prostate cancer, FAK is known primarily for its role in cell motility and cytoskeletal rearrangement, as supported by *in vivo* and *in vitro* evidence (15). PCNA is a nuclear protein synthesized in the G1/S phase and plays a significant role in DNA replication. PCNA expression is low in non-dividing cells, but increases greatly in proliferating and transformed cells. In the present study, anti-FAK-pY397-specific (Biosource, Camarillo, CA, USA) and anti-PCNA-specific (Abcam) antibodies were used. The expression of FAK and PCNA in groups A and B was higher than that in group C (Fig. 2A, B, C, J, K and L).

## Discussion

Recent studies have focused on the role of TRPM8, rendering it a novel molecular target potentially useful in the diagnosis and treatment of PC. The channel is activated by voltage, cold temperatures and cooling compounds, such as menthol and icilin (16). Our previous results indicated that the overexpression of TRPM8 has a negative effect on the proliferation and malignant progression of PC-3 cells *in vitro* (7). Similarly, Gkika *et al* (17) have demonstrated that PC-3 cells artificially overexpressing TRPM8 have reduced motility, suggesting a possible connection between TRPM8 activity and reduced metastatic potential. In this study, the tumor volume in group C was less than that in groups A and B, suggesting that the overexpression of TRPM8 possibly has a negative effect on the proliferation of PC-3 cells *in vivo*.

It is well established that the growth and dissemination of solid tumors is dependent on angiogenesis (18). Human VEGF mRNA is transcribed from eight exons of a single gene and is

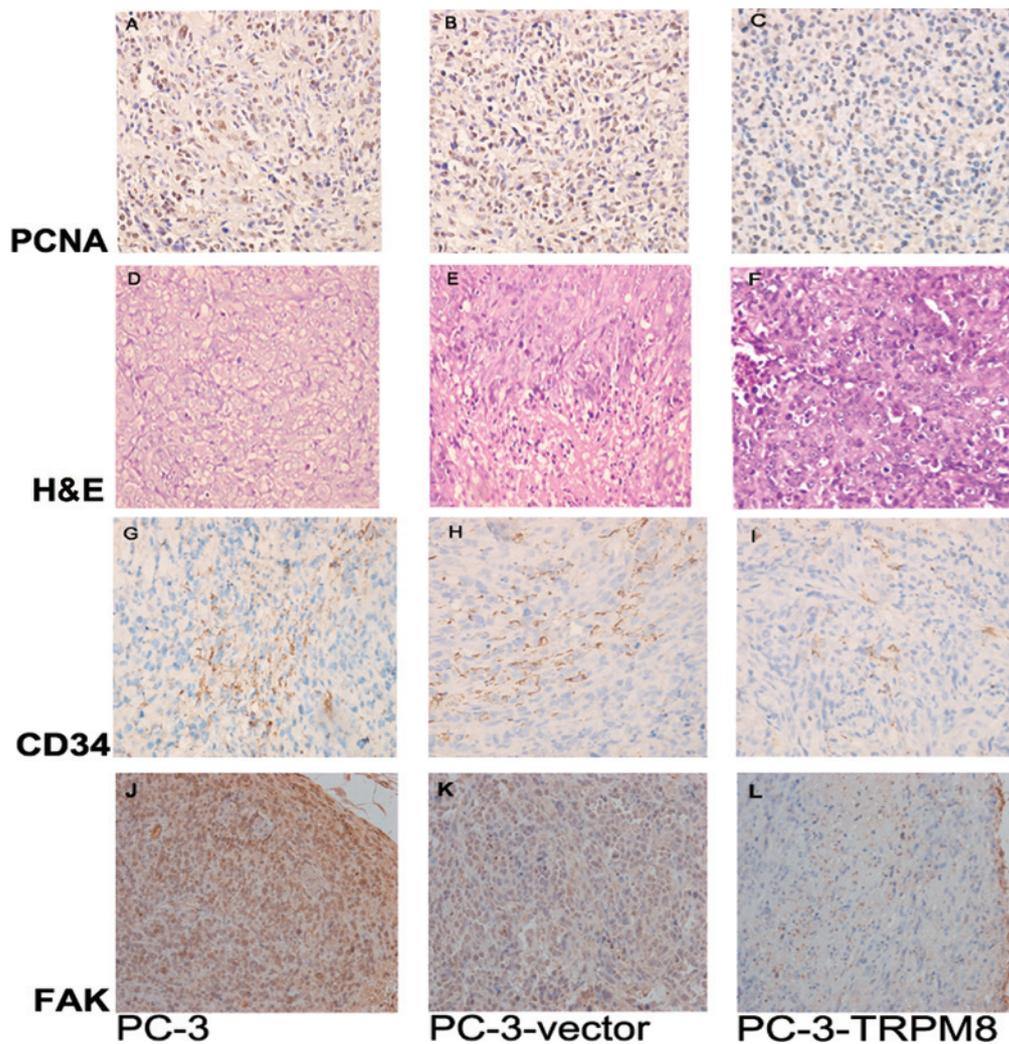


Figure 2. H&E, PCNA, CD34 and FAK staining of tumor tissue (magnification, x200). (A-C) PCNA expression in tumor tissue; compared to the PC-3 and PC-3-vector groups (A and B), PCNA expression was decreased in the PC-3-TRPM8 group (C). (D-F) H&E staining of tumor tissue. There was no difference in Gleason score among the three groups. (G-I) Blood vessels were stained using anti-CD34 antibody; MVD of the PC-3 and PC-3-vector groups (G and H) was higher than that of the PC-3-TRPM8 group (I) ( $P<0.05$ ). (J-L) FAK expression in tumor tissue; compared to the PC-3 and PC-3-vector groups (J and K), FAK expression was decreased in the PC-3-TRPM8 group (L).

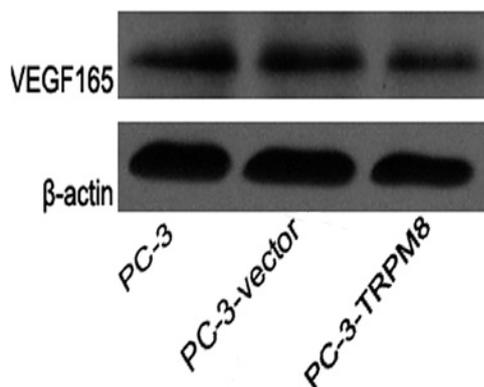


Figure 3. The PC-3, PC-3-vector and PC-3-TRPM8 groups exhibit VEGF protein expression. Expression of VEGF protein in the PC-3 and PC-3-vector groups was higher than that in the PC-3-TRPM8 group ( $P<0.05$ ).

alternatively spliced into at least six mRNAs, which give rise to the mature proteins of 121, 145, 165, 183, 189 and 206 amino acids. VEGF<sub>121</sub> and VEGF<sub>165</sub> are the best characterized and are

the most abundant in normal tissues, including blood vessels. As with most tumors, prostate tumors overexpress VEGF, thereby promoting the development of tumor neovascularization (19). Certain studies using immunohistochemistry have reported an increased expression of total hVEGF protein in human prostate tumors, when compared with normal tissue or preinvasive prostate lesions (20). Our results revealed protein expression of VEGF<sub>165</sub> in each group, but VEGF<sub>121</sub> was not detected. This lack of detection may be because VEGF<sub>165</sub> has a greater molecular weight and is expressed more widely. Furthermore, in this study, the VEGF expression level of group C was lower than that of groups A and B ( $P=0.00$  and  $P=0.00$ , respectively).

MVD is a prognostic marker for various tumors, including prostate cancer (21). In prostate cancer, MVD is correlated with the development of metastases, clinical stage and overall patient survival (22). In addition, the progression of prostate cancer into the AI state has been shown to be associated with increased angiogenesis (23); thus, antiangiogenic therapy may

be a possible means of improving treatment for patients with HRPC. MVD is a quantitative description of angiogenesis. In this study, the MVD of group C was significantly decreased compared to that of groups A and B ( $P=0.045$  and  $P=0.041$ , respectively; Fig. 2G, H and I; Table II), which, coupled with the results of the expression of VEGF, indicated that TRPM8 may have a negative effect on the angiogenesis of PC-3 cells *in vivo*.

PCNA is a nuclear protein and plays a significant role in DNA replication. The expression level of PCNA is closely correlated to the cell state, which means that the level of PCNA expression correlates with the degree of malignancy, invasion and metastasis in cancer cells. Thus, PCNA is a significant evaluative marker for tumor growth and prognosis (24). FAK is a non-receptor protein tyrosine kinase that regulates adhesion-dependent cell signaling (14). FAK expression is increased in prostate cancer cell lines (25), and an increased expression correlates with enhanced motility and tumorigenicity (26). Our previous study indicated that overexpression of TRPM8, through inactivation of FAK, reduced the motility of PC-3 cells *in vitro* (7). In this study, we have further shown that TRPM8 inhibits the expression of FAK *in vivo*. The results of this study have shown that the expression of FAK and PCNA in group C was lower than that in groups A and B (Fig. 2J, K and L). Coupled with the results of tumor volume in each group, the TRPM8 channel may mediate the repair and synthesis of DNA, and may, through inactivation of FAK, have a negative effect on proliferation. However, there was no difference in the Gleason score of each group, and the reason for this remains to be determined, but possibly lies in the limited time taken for the graft to grow.

In conclusion, this study demonstrates that the overexpression of TRPM8 had a negative effect on the proliferation and angiogenesis progression of PC-3 cells *in vivo*. Therefore, for patients in the AI 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 and should be further investigated.

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