TRPV6 channel controls prostate cancer cell proliferation via Ca(2+)/NFAT-dependent pathways.

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CANCER CELL PROLIFERATION VIA Ca^{2+}/NFAT-DEPENDENT PATHWAYS

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Abstract

The transient receptor potential channel, subfamily V, member 6, TRPV6 is strongly expressed in advanced prostate cancer and significantly correlates with the Gleason >7 grading, as it is undetectable in healthy tissue. However, the role of TRPV6 as a highly Ca^{2+}-selective channel in prostate carcinogenesis was not clearly understood. Here, we report that TRPV6 is directly involved in LNCaP cell proliferation. Indeed, TRPV6 knockdown significantly inhibited cell growth by decreasing: (i) proliferation rate; (ii) accumulation of S-phase cells; and (iii) proliferating cell nuclear antigen expression. Ca^{2+}-entry into LNCaP cells upon acute changes in $[Ca^{2+}]_o$ from 0 to 2 mM was completely blocked by siRNA-TRPV6, suggesting that TRPV6 mediates Ca^{2+}-uptake in these cells. Co-transfection of LNCaP cells with the pNFAT-Luciferase vector led to a significant decrease in NFAT promoter activation following siRNA-TRPV6 treatment. TRPV6 knockdown also increased apoptosis in ~15% of LNCaP cells suggesting its role in apoptosis resistance to approximately 50%, compared to siRNA-AR treatment. Ca^{2+}-entry into LNCaP cells upon acute changes in $[Ca^{2+}]_o$ from 0 to 2 mM was completely blocked by siRNA-TRPV6, suggesting that TRPV6 is constitutively open and mediates Ca^{2+}-uptake in these cells . Co-transfection of LNCaP cells with the pNFAT-hrGFP vector led to a significant decrease in NFAT promoter activation following siRNA-TRPV6 treatment. Androgen receptor knockdown dramatically decreased TRPV6 levels, which were relatively insensitive tonot significantly affected by DHT/Casodex treatment, suggesting that AR is indirectly involved in regulation of TRPV6 expression in ligand-independent fashion. We conclude that expression of the TRPV6 Ca^{2+}-channel by the cancer cell may represent a mechanism for maintaining a higher proliferation rate, as well as increasing cell survival and apoptosis resistance.

Prostate cancer remains the most common noncutaneous human malignancy and the second most lethal tumor among men with the highest incidence in industrialized countries (Cooperberg et al., 2005). Androgen receptor (AR) and cognate ligands regulate vital aspects of prostate cell growth and functions, including proliferation, differentiation, apoptosis, and secretion (Burnstein, 2005). The AR pathway also influences prostate pathological processes, such as benign prostatic hyperplasia and carcinogenesis. Standard therapies for prostate cancer include androgen ablation, which causes tumor regression. However, under anti-androgen therapy, prostate cancer and metastases progress into an androgen-independent stage, causing cancer relapse with a more aggressive phenotype. Therefore, it is critical to understand the mechanisms involved in prostate cancer progression to develop reliable prognostic markers and define new treatment strategies.

It has been previously suggested that intracellular calcium is a second messenger playing a major role in the control of cell proliferation, differentiation and apoptosis. Indeed, The function of ion channels has been discussed in relation to proliferation and apoptosis.The role of calcium (Ca^{2+}) in global cancer-related cell signaling pathways is uncontested.a Alterations in Ca^{2+} homeostasis increase proliferation (Legrand et al., 2001; Thebault et al., 2006), as well as induceinging differentiation (Vanoverberghe et al., 2004) and apoptosis (Skryma et al., 2000; Vanden Abeele et al., 2003; Vanden Abeele et al., 2002). According to a growing number of articles, cationic channels in the TRP (Transient Receptor Potential) family are key players in calcium homeostasis and cell physiopathology (Montell et al., 2002). In recent years, TRPV6 (TRP, Vanilloid member 6), a highly Ca^{2+} selective channel, has emerged as a promising prognosis marker. TRPV6 is strongly expressed in advanced prostate cancer and significantly correlates with the Gleason >7 grading, making it a strong marker of tumor progression and subsequent invasion into healthy tissues (Fixemer et al., 2003; Wissenbach et al., 2004). Previous studies have shown that TRPV6 is involved in highly calcium selective currents in prostate cells and that it is tightly regulated by intracellular Ca^{2+} concentrations ($[Ca^{2+}]_i$) (Bodding & Flockerzi, 2004; Schindl et al., 2002; Vanden Abeele et al., 2004). However, the precise role of Ca^{2+} entry *via* TRPV6 in prostate physiopathology remains illusive. Furthermore, reports on its regulation by androgen are contradictory (Peng et al., 2001).

We initially studied TRPV6 protein expression in the LNCaP human prostate cancer cell line. Figure 1A shows detection of the expected 84 kDa TRPV6 protein. TRPV6 expression level decreased significantly after 4 days' siRNA transfection (Fig. 1A). In order to study the relative contribution of TRPV6, as a highly Ca^{2+}-selective channel, to Ca^{2+}-uptake in LNCaP cells, we measured $[Ca^{2+}]_i$ in LNCaP cells after substantial changes in $[Ca^{2+}]_o$. In control cells, variations in $[Ca^{2+}]_o$ produced significant changes in $[Ca^{2+}]_i$ (Fig. 1B). siRNA-TRPV6 knockdown decreased the amplitude of the 2 mM $[Ca^{2+}]_o$-evoked $[Ca^{2+}]_i$. increase. These data strongly suggest that TRPV6 is constitutively open and mediates Ca^{2+}-uptake in LNCaP cells. Recent research has demonstrated that transfecting HEK-293 cells with TRPV6 increases the Ca^{2+}-dependent cell proliferation rate, but the role of endogenous TRPV6 in

prostate cancer cell growth control remains unknown (Schwarz et al., 2006). It was, therefore, interesting to study the potential involvement of TRPV6 in LNCaP cell growth in greater depth. The number of viable proliferating cells, measured by MTS assay, decreased significantly following TRPV6 knockdown from day 2 to 4 after transfection (D0) (Fig. 1C). siRNA against AR, known to be crucial for prostate growth and development (Burnstein, 2005), was used as a positive control. We also performed a cell cycle assay with propidium iodide staining to clarify TRPV6 knockdown effects on the cell-cycle phase distribution of LNCaP cells (Fig. 1D). Indeed, siRNA-TRPV6 decreased the number of cells entering the S-phase (siRNA-AR, used as positive control, evoked the same decrease). To demonstrate the specificity of the studied effects ascribed to TRPV6, we performed the silencing of the other TRP-channel in the plasma membrane, TRPC3, which had no significant effects on both cell proliferation and cell cycle (Fig. 1C, D, F). Also, To confirm these results, wwe monitored the protein level of proliferating cell nuclear antigen (PCNA). Fig. 1E shows one order decrease in that the PCNA level seemed to decrease significantly following siRNA-TRPV6 treatment, whereas it wasbeing almost undetectable in siRNA-AR-treated cells. It was previously shown that human primary prostate cancer epithelial cell proliferation was promoted by store-independent Ca^{2+} entry and subsequent activation of nuclear factor of activated T cell (NFAT) transcription factor (Thebault et al., 2006). Interestingly, co-transfecting LNCaP cells with siRNA-TRPV6 and pNFAT-hrGFP Luciferase plasmid revealed that TRPV6-induced Ca^{2+} entry made a considerable contribution in activating the promoter of Ca^{2+}-dependent NFAT transcription factor (Fig. 1FG), known to be involved in Ca^{2+}-dependent cell proliferation (Lipskaia & Lompre, 2003). Thus, TRPV6 is most probably involved in LNCaP cell proliferation by mediating Ca^{2+}-entry, followed by the activation of Ca^{2+}-dependent NFAT transcription factor - mediated signaling pathways.

It is also well established that $[Ca^{2+}]_i$ increase favors cell proliferation whereas sustained $[Ca^{2+}]_i$ increase, with the subsequent mitochondrial overload, has pro-apoptotic potential (for review see: (Prevarskaya et al., 2004)). In this study, we investigated whether Ca^{2+} entry *via* TRPV6 was pro- or anti-apoptotic in cancer cells. A cell-cycle assay measured the number of apoptotic cells as a subG1 population. siRNA-TRPV6 pre-treatment significantly increased the number of apoptotic cells (~15%) as compared to ~35% induced by siRNA-AR (Fig. 1G1F). Thus, our data indicate that TRPV6-expression promotes apoptosis resistance in the LNCaP cell line.

Therefore, Ca^{2+} entry *via* TRPV6 may not only be a mechanism for maintaining a high proliferation rate but also for increasing cell survival and inducing apoptosis resistance.

As, on the one hand, TRPV6 has previously been shown to be overexpressed in high-grade prostate cancer (Fixemer et al., 2003; Wissenbach et al., 2004) and, on the other hand, prostate cancer development is androgen-dependent (Burnstein, 2005), we investigated whether *trpv6* gene regulation was under AR control. Three approaches were used to study TRPV6 regulation by androgen in the LNCaP human, cancerous, androgen-responsive cell line: (i) siRNA-AR; (ii) AR selective ligand

dihydrotestosterone (DHT, 1 nM); (iii) and AR selective antagonist Casodex (10 µM). Thiese approaches wasere previously used to demonstrate a direct positive regulation by AR of TRPM8, another channel in the TRP family (Bidaux et al., 2005). In this study, AR knockdown by 200 nM siRNA significantly decreased TRPV6 mRNA after 48 h and protein levels 72 h after post- transfection (Fig. 2A, B). These data were confirmed by immunocytochemistry (Fig. 2CH). In the series of Ca^{2+}-imaging experiments we tried to see studied whether Ca^{2+}-uptake into LNCaP cell was affected by siRNA against AR. Fig. 2C shows that Ca^{2+}-uptake is substantially suppressed upon AR knockdown which is consistent with the fact that TRPV6 represents the major Ca^{2+}-uptake channel in the prostate cancer cell. Taken together Tthese results suggested that TRPV6 knockdown was amay be reproduced by consequence of AR downregulationregulates the TRPV6 expression and/or activity. and therefore *trpv6* gene expression is regulated by AR. On the contrary, AR expression was not dependent on *TRPV6* gene regulation. In the nextTo elucidate the nature of this regulation stage, LNCaP cells grown in 2% serum-supplied RPMI medium were treated with either 1 nM DHT AR selective agonist or 10 µM Casodex selective antagonist for 24 h (Fig. 2DCD). Both DHT and Casodex had no significant effects on TRPV6 expression, whereas Casodex downregulated TRPV6 mRNA expression (Fig. 2E). DHT/Casodex effects were already studied in the context of androgen regulation of TRPV6 (Peng et al., 2001; Bodding et al., 2003; Vanden Abeele et al., 2003). We believe that these variations in TRPV6 expression were too weak (e.g. 0.6 and 1.5 fold changes upon DHT/Casodex treatments, respectively (Peng et al., 2001)) to suggest any ligand-dependency of AR regulation of *trpv6* gene as compared to known androgen regulation of other TRP channel, e.g. TRPM8 in LNCaP (5-6 fold increase upon DHT treatment (Bidaux et al., 2005)). This result was concomitant with AR downregulation. The absence of consistency/reproducibility in agonist/antagonist treatments had led us Toto check whether the functional blockade of AR affected TRPV6 expression,. Thus, we applied aandrogen deprivation for 6 days was used. In LNCaP cells, this treatment led to only a gradual1.8-fold increase in TRPV6 protein expression levels. Variations in AR protein levels are presented in Fig. 2EDEF. Taken the role of AR in differentiation of LNCaP cells upon androgen deprivation (Vanoverberghe et al., 2004), it is likely that AR preserves its functionality. In view of the role played by AR in LNCaP cell differentiation following androgen deprivation (Vanoverberghe et al., 2004), AR probably remained functional. The addition of 1 nM DHT to LNCaP cells deprived of steroids for 6 days did not lead to any significant change in either TRPV6 or AR mRNA expression levels. However, the apparent upregulation of PSA following DHT treatment constituted a positive control for the experiment (Fig. 2FEFG). The fact that the functional status of AR, i.e. transcriptional activity, strongly correlates with its phosphorylation status other than activation by androgens, has already been considered for the treatment of prostate cancer (Wang et al., 1999). It is interesting to note that in 2001 the first effort was made to define androgen regulation of TRPV6 (Peng et al., 2001). The authors reported 60% and 150% variations in DHT and Casodex effects on *trpv6* gene expression, respectively. In the other work 70% and 30% variations in DHT and Casodex effects, respectively, were

reported (Bodding et al., 2003). We believe that these variations in TRPV6 expression could not suggest any ligand-dependency of AR regulation of *trpv6* gene. Increasing evidence indicates that androgens may also have an indirect influence on the expression of genes that do not contain AR-responsive elements by modulating the activity of other transcription factors, mediating growth factor expression in paracrine or autocrine mode, or inducing changes in the production of other hormones (Korkmaz et al., 2002; Xiao et al., 2005). We chose another steroid receptor, VDR, as a positive model of TRPV6 regulation, since *trpv6* has already been identified as a VDR-regulated gene (Wang et al., 2005), and it seems to activate the *trpv6* gene directly in LNCaP cells (Fig. 2GH). Unlike DHT, 1,25-dihydroxyvitamin D3 increased TRPV6 mRNA expression in a steroid-containing RPMI medium in a dose-dependent manner (Fig. 2I).

Taken together, these data on *trpv6* gene regulation suggest that *trpv6* is not a direct AR-responsive gene, however the presence of AR is needed in this cell type to maintain the expression of TRPV6. We conclude that . However, as itTRPV6 is indirectly regulated by AR in ligand-independent fashion and probably constitutes an essential co-factor of *trpv6* gene transcription in prostate cancer cells., it represents an essential component in the aggressiveness of prostate cancer cells.

In summary, this research demonstrated for the first time that Ca^{2+} entry *via* TRPV6 controlled proliferation directly and promoted apoptosis resistance in prostate cancer cells. In view of TRPV6's strong correlation with the Gleason >7 tumor grading in prostate cancer, this channel is a promising therapeutic target for the development of new treatments.

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Titles and Legends to Figures

Fig. 1 Ca^{2+} *via* TRPV6 is involved in LNCaP cell proliferation. **A:** A representative western-blot of TRPV6 was performed on LNCaP cells as compared to β-actin. LNCaP cells were lysed and the lysates were centrifuged at 15,000 × g and 4°C for 20 minutes, mixed with Laemmli buffer and boiled at 95°C for 5 min. Total protein samples were subjected to 8% SDS-PAGE and transferred to a nitrocellulose membrane by semi-dry Western blotting (Bio-Rad Laboratories). The membrane was probed using rabbit polyclonal anti-TRPV6 (Alomone Labs LTD, 1/100) and anti-β-actin (Lab Vision Co., 1/1000) antibodies followed by secondary antibodies. The bands on the membrane were displayed using enhanced chemiluminescence method (Pierce Biotechnologies Inc.). Densitometric analysis was performed using a Bio-Rad image acquisition system (Bio-Rad Laboratories). **B**: The effects of TRPV6 inhibition on Ca^{2+} entry into LNCaP cells. LNCaP cells were plated onto glass coverslips and transfected with functional non-coding siRNA#1 as a control and siRNA-TRPV6. Cells were loaded with 4 μM Fura-2 AM in the growth medium at room temperature for 45 min. $[Ca^{2+}]_i$ was measured after subsequent $[Ca^{2+}]_o$ switches, as indicated.. n=150 cells for each condition. **C**: Cell proliferation of LNCaP cells measured using the CellTiter 96 Aqueous One Solution cell proliferation assay (Promega). LNCaP cells were plated in 96-well plate and transfected using "Gene porter 2" (Gene Therapy Systems, Inc.) reagents, with a functional non-coding siRNA#1 (Dharmacon Research Inc.) as a control, siRNA-TRPV6 (siTRPV6 (5'-CCUGCUGCAGCAGAAGAGGTT-3'), 200 nM, transfected at D0), siRNA-TRPC3 (siTRPC3 (5'-UGAUGUGGUCUGAAUGUAA(dTdT)-3'), 200 nM, transfected at D0), and siRNA-AR (siAR (5'-GACUCAGCUGCCCCAUCCATT-3'), 200 nM, transfected at D0) as a positive control. Asterisks denote statistical significance as compared to control cells, * - $P<0.05$, ** - $P<0.01$; n=4; **D**: a cell cycle distribution of LNCaP cells treated as above, carried out by flow cytometry of the cells stained with propidium iodide and analyzed within 2 h on a FACScan flow cytometer (Becton–Dickinson, San Jose, CA). Asterisks denote statistical significance as compared to control cells, * - $P<0.05$, ** - $P<0.01$; n=3. **E:** A western-blot of proliferating cell nuclear antigen (PCNA) Membrane was probed using mouse monoclonal anti-PCNA (Santa-Cruz, 1/1000) and anti-β-actin (Lab Vision Co., 1/1000) antibodies followed by secondary antibodies; n=3. **F**: An apoptosis assay carried out by flow cytometry, as described above, on a subG1 population of LNCaP cells. Asterisks denote statistical significance, * - $P<0.01$ vs. control, ** - $P<0.01$ vs. siTRPV6 treatment; n=3.Ca^{2+}-entry via TRPV6 is involved in the activation of Ca^{2+}-dependent transcriptional factor, NFAT. Cells were co-transfected with a functional non-coding siRNA#1 and pNFAT-hrGFP vector (Stratagene), pNFAT-hrGFP vector and siTRPV6 and siAR, respectively. hrGFP was detected by western-blotting using rabbit polyclonal anti-GFP antibody (Abcam) and the comparative histogram shows the effects of TRPV6 on NFAT signaling. * - $P<0.05$; ** - $P<0.01$ vs. CT+vector, n=3. **G**: Ca^{2+}-entry via TRPV6 is involved in the activation of Ca^{2+}-dependent

transcriptional factor, NFAT. Cells were co-transfected with a functional non-coding siRNA#1 and pNFAT-Luciferase vector (Promega), pNFAT-Luciferase vector and siTRPV6 and siAR, respectively. In addition, all the cells were pre-transfected with β-galactosidase reporter vector (Promega) to normalize the results. The comparative histogram shows the effects of TRPV6 on NFAT signaling. * - P<0.01; vs. CT+vector, n=3.An apoptosis assay carried out by flow cytometry, as described above, on a subG1 population of LNCaP cells. Asterisks denote statistical significance, * - P<0.01 vs. control, ** - P<0.01 vs. siTRPV6 treatment; n=3.

Fig. 2 TRPV6 is an indirect androgen-dependent gene. The effects of AR knockdown on: (**A**) mRNA and (**B**) protein levels in TRPV6, and AR itself, compared to β-actin. **C**: The effects of AR silencing on Ca^{2+} entry into LNCaP cells. LNCaP cells were plated onto glass coverslips and transfected with functional non-coding siRNA#1 as a control and siRNA-AR. Cells were loaded with 4 μM Fura-2 AM in the growth medium at room temperature for 45 min. $[Ca^{2+}]_i$ was measured after subsequent $[Ca^{2+}]_o$ switches, as indicated. n=150 cells for each condition. **D**: The effects of DHT AR agonist (1 nM) and Casodex AR antagonist (Csdx, 10 μM) for 1 day on TRPV6 and AR mRNA levels as compared to β-actin. **Immunocytochemistry of TRPV6 and AR in LNCaP cells following reciprocal knockdown by siAR and siTRPV6. Cells grown on glass coverslips were fixed in 3.5% paraformaldehyde in PBS, then washed in PBS and stained with both rabbit polyclonal anti TRPV6 antibody (Alomone Labs Ltd., 1/200) and mouse monoclonal anti-AR (Neomarkers, 1/500). Alexa Fluor® 546 goat anti-rabbit IgG (Molecular Probes, 1/4000) was used as a secondary antibody for TRPV6 staining, and Alexa Fluor® 488 donkey anti-mouse IgG (Molecular Probes, 1/4000) for AR staining. Fluorescence analysis was carried out using a Carl Zeiss Laser Scanning Systems LSM 510 connected to a Zeiss Axiovert 200 M with 63X1.4 numerical aperture oil immersion lens at room temperature, and data were processed using Carl Zeiss LSM Image Examiner software. DE**: The corresponding histogram of the DHT/Casodex effects on TRPV6 mRNA expression in LNCaP cells. **F:** Effects of steroid deprivation (SD) on TRPV6 and AR protein levels as compared to β-actin. **The effects of DHT AR agonist (1 nM) and Casodex AR antagonist (Csdx, 10 μM) for 1 day on TRPV6 and AR mRNA levels as compared to β-actin. EG:F:** Effects of 1 nM DHT on TRPV6, AR, and PSA as a control, LNCaP cell mRNA expression levels in LNCaP cell line after 6 days of steroid deprivation. Effects of steroid deprivation (SD) on TRPV6 and AR protein levels as compared to β-actin. **F:** Effects of 100 nM 1,25-dihydroxyvitamin D3 on TRPV6 mRNA level in LNCaP cells.Effects of 1 nM DHT on TRPV6, AR, and PSA as a control, LNCaP cell expression levels after 6 days of steroid deprivation . **H: G:** Immunocytochemistry of TRPV6 (red) and AR (green) in LNCaP cells following reciprocal knockdown by siAR and siTRPV6. Cells grown on glass coverslips were fixed in 3.5% paraformaldehyde in PBS, then washed in PBS and stained with both rabbit polyclonal anti TRPV6 antibody (Alomone Labs Ltd.,

1/200) and mouse monoclonal anti-AR (Neomarkers, 1/500). Alexa Fluor® 546 goat anti-rabbit IgG (Molecular Probes, 1/4000) was used as a secondary antibody for TRPV6 staining, and Alexa Fluor® 488 donkey anti-mouse IgG (Molecular Probes, 1/4000) for AR staining. Fluorescence analysis was carried out using a Carl Zeiss Laser Scanning Systems LSM 510 connected to a Zeiss Axiovert 200 M with 63X1.4 numerical aperture oil immersion lens at room temperature, and data were processed using Carl Zeiss LSM Image Examiner software. **HI:** Effects of 100 nM 1,25-dihydroxyvitamin D3 on TRPV6 mRNA level in LNCaP cells. Effects of 100 nM 1,25-dihydroxyvitamin D3 on TRPV6 mRNA level in LNCaP cells. PCR: Total RNA was isolated using the guanidium thiocyanate-phenol-chloroform extraction procedure, reverse transcribed using MuLV reverse transcriptase (Perkin Elmer). The following primers were used for PCR: TRPV6 (NM_018646), F1: ATGGTGATGCGGCTCATCAGTG; B1: GTAGAAGTGGCCTAGCTCCTCG; AR (NM_000044), F1: TGTCCATCTTGTCGTCTTCG; B1: CTGGAGTTGACATTGGTGAAGG; PSA (NM_001648), F1: CTCACCCTGTCCGTGACGTGGATT; B1: AAGCTGTGGCTGACCTGAAATA; β-Actin (NM_001101), F1: CAGAGCAAGAGAGGCATCCT; B1: GTTGAAGGTCTCAAACATGATC. DNA amplification conditions included an initial 5 min denaturation step at 95° C, and 33 cycles of 30 sec at 95° C, 30 sec at 59° C, 30 sec at 72° C, and finally 7 min at 72° C. All experiments were performed at least 3 times unless otherwise stated. Both PCR and western blotting bands were quantified using densitometric analysis option in the Bio-Rad image acquisition system (Bio-Rad Laboratories). See Fig. 1 for western-blotting specifications.See Fig. 1 for western-blotting specifications.