RNA interference-mediated depletion of TRPM8 enhances the efficacy of epirubicin chemotherapy in prostate cancer LNCaP and PC3 cells

TAO LIU[1*], YIXIANG LIAO[1*], HUANGHENG TAO[2*], JINMIN ZENG[1], GANG WANG[3], ZHONGHUA YANG[3], YONGZHI WANG[3], YU XIAO[3], JIAJIE ZHOU[1] and XINGHUAN WANG[3]

[1]Department of Urology, Jingzhou Central Hospital, The Second Clinical Medical College, Yangtze University, Jingzhou, Hubei 434020; [2]Key Laboratory for Oral Biomedicine of Ministry of Education, School and Hospital of Stomatology, Wuhan University; [3]Department of Urology, Center for Medical Science Research, Zhongnan Hospital of Wuhan University, Wuhan, Hubei 430071, P.R. China

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Abstract. Several studies have shown that transient receptor potential cation channel subfamily M member 8 (TRPM8), which has been regarded as a novel prostate-specific marker, serves a key role in processes such as the proliferation, viability and cell migration of prostate cancer cells. Efforts have been made to uncover the potential role of targeting TRPM8 in the management of prostate cancer; it has been verified that TRPM8-targeted blockade, either by RNA interference-mediated depletion or specific TRPM8 inhibitors, could reduce the rate of proliferation and proliferative fraction, and induce apoptosis in prostate cancer cells. The aim of the present study was to investigate the effect of knockdown of TRPM8 on chemosensitivity in prostate cancer LNCaP and PC3 cells. The RNA interference-mediated depletion of TRPM8 inhibited proliferation and enhanced epirubicin chemosensitivity of LNCaP and PC3 cells, and promoted epirubicin-induced apoptosis by increasing the phosphorylation of p38 mitogen-activated protein kinase (hereafter p38) and c-Jun N-terminal kinase (JNK)/mitogen-activated protein kinase signaling pathways, which was demonstrated via the use of specific inhibitors of phosphorylation of p38 and JNK. The results demonstrate that the targeted silencing of TRPM8 expression is a therapeutic strategy for treatment of prostate cancer that has considerable potential, even for castration-resistant prostate cancer.

Correspondence to: Professor Jiajie Zhou, Department of Urology, Jingzhou Central Hospital, The Second Clinical Medical College, Yangtze University, 72 Jingzhong Road, Jingzhou, Hubei 434020, P.R. China
E-mail: zhoujiajiejingzhou@126.com

Professor Xinghuan Wang, Department of Urology, Center for Medical Science Research, Zhongnan Hospital of Wuhan University, 169 Donghu Road, Wuhan, Hubei 430071, P.R. China
E-mail: wangxinghuan@whu.edu.cn

*Contributed equally

Abbreviations: TRPM8, transient receptor potential cation channel subfamily M member 8; EPI, epirubicin; PCa, prostate cancer; siRNA, small interference RNA; siTRPM8, siRNA targeting TRPM8; siCON, negative control scrambled siRNA; CRPC, castration-resistant prostate cancer

Key words: RNA interference, transient receptor potential cation channel subfamily M member 8, epirubicin, chemotherapy, prostate cancer

Introduction

Prostate cancer (PCa) is one of the leading threats to men's health, accounting for 25% of incident cases of cancer diagnosed in adult males in the United States annually, and ~9% of incidences of cancer-associated mortality in the same population, according to statistical data from 2016 (1). PCa cells are considered to depend on androgens for survival and growth in its early stages (2), and androgen ablation therapy may be a sensitive and effective way of reducing tumor growth; however, the treatment options for the late-stage disease termed castration-resistant prostate cancer (CRPC), remain relatively inefficient (3), as CRPC responds poorly to chemotherapy (4). Epirubicin (EPI) has been repeatedly found to exhibit activity as a cytotoxic agent for prostate cancer patients, either administered alone or in combination; however, drug resistance often leads to treatment failure in CRPC patients (5).

As a non-selective Ca^{2+}-permeable cation channel, transient receptor potential cation channel subfamily M member 8 (TRPM8) serves a key role in Ca^{2+} homoeostasis, which is one of the vital factors in cancer-associated cell signaling pathways. Fluctuations in Ca^{2+} homeostasis may lead to an increase in cell proliferation (6,7), migration (8) and may even induce differentiation and apoptosis (9-12). Therefore, TRPM8 has emerged as a promising prognostic marker and putative therapeutic target in PCa for its vital

role in Ca^{2+} transportation (13-21). TRPM8 is abundantly expressed in the prostate, an expression that increases in PCa, which indicates that there exists the potential to treat prostate cancer via the specific gene-silencing of TRPM8. Zhang and Barritt (2) reported that TRPM8-knockdown could lead to the suppression of LNCaP cell proliferation. Studies by Valero *et al* (17,22) indicated that the small interfering RNA (siRNA) inhibition of TRPM8 expression or small molecule inhibition of function using the specific TRPM8 blockers *N*-(3-aminopropyl)-2-{[(3-methylphenyl) methyl] oxy}-*N*-(2-thienylmethyl)benzamide (AMTB) and JNJ41876666 reduced the rate of proliferation and proliferative fraction in prostate cancer cells, but not in normal prostate cells. A previous study demonstrated that *N*-(4-tertiarybutylphenyl)-4-(3-chloropyridin-2-yl) tetrahydropyrazine-1(2H)-carboxamide BCTC, a potent and representative antagonist of TRPM8, exerted an antitumor effect on androgen-independent prostate cancer DU145 cells by altering levels of phosphorylated RAC serine/threonine-protein kinase (p-AKT), cyclin D1, cyclin-dependent kinase 2 (CDK2), CDK6, p-glycogen synthase kinase-3β (p-GSK-3β) and proteins involved in the mitogen-activated protein kinase (MAPK) signal pathways (23). Further, whether the knockdown of TRPM8 influence on the chemosensitivity of prostate cancer was assessed in the present study. The anthracycline EPI, either alone or in combination with other agents, has been extensively used in the treatment of CRPC; however, chemoresistance increases as the duration of treatment extends (24). The present study investigated the potential effect of TRPM8-knockdown on the chemosensitivity to EPI of prostate cancer cells. The present study reports evidence that the knockdown of TRPM8 enhanced the chemosensitivity of prostate cancer cells to EPI, which would indicate the potential of a targeted TRPM8-silencing therapeutic strategy to cure of PCa. To determine the potential efficacy of this treatment approach, cell and molecular analyses were performed following the silencing of the TRPM8 gene in prostate cancer LNCaP and PC3 cells using a siRNA. The results of these analyses revealed that the silencing of TRPM8 effectively inhibited cellular proliferation, yet failed to induce apoptosis in LNCaP and PC3 cells. Nevertheless, siRNA treatment of TRPM8 increased EPI-induced apoptosis in LNCaP and PC3 cells. Silencing of TRPM8 was accompanied by the upregulation of p38 MAPK (hereafter p38) and c-Jun N-terminal kinase (JNK) phosphorylation, and ultimately results in the increased sensitivity of PCa cells to EPI. Taken together, these results indicate that TRPM8 may represent an effective target for the treating CRPC.

Materials and methods

Cell culture. All the prostate cancer cell lines, LNCaP, DU145, PC3, and the non-cancer cell line PNT1A were obtained from the American Type Culture Collection (Manassas, VA, USA). All the cells were cultured in RPMI-1640 medium (Gibco; Thermo Fisher Scientific, Inc., Waltham, MA, USA) containing 10% fetal bovine serum (FBS, Gibco; Thermo Fisher Scientific, Inc.), 100 U/ml penicillin-G sodium (Sigma-Aldrich; Merck KGaA, Darmstadt, Germany) and 100 mg/ml streptomycin sulfate (Sigma-Aldrich; Merck KGaA) at 37˚C in a humidified atmosphere containing 5% CO_2.

RNA interference-mediated gene silencing of TRPM8. The specific siRNA sequence targeting TRPM8 (siTRPM8) was 5'-UCUCUGAGCGCACUAUUCA(dTdT)-3' (1); the sequence of negative control scrambled siRNA (siCON) was 5'-UUCUCCGAACGUGUCACGUTT-3' (not homologous to any gene). These siRNAs were synthesized by Shanghai GenePharma Co., Ltd. (Shanghai, China). The LNCaP or PC3 cells were seeded into 6-well plates at 30-40% confluence and cultured in 2 ml of RPMI-1640 medium containing 10% FBS until the cells reached 70% confluence. The siRNA-Lipofectamine 2000 (Thermo Fisher Scientific, Inc.) complex was pre-mixed (10 nM siTRPM8 or siCON and 8 μl Lipofectamine reagent per well) according to the manufacturer's instructions and added to the 6-well plates. Cells transfected with siTRPM8 and siCON are termed siTRPM8-LNCaP and siCON-LNCaP or siTRPM8-PC3 and siCON-PC3 cells, respectively; untransfected cells are termed parental LNCaP or PC3 cells. The expression of LNCaP or PC3 cells transfected with siTRPM8 and siCON was evaluated by RT-PCR and western blot analysis. EPI was added once cells had been transfected for 24 h.

Reverse transcription-polymerase chain reaction (RT-PCR). Total RNA was isolated from the parental PNT1A, DU145, LNCaP and PC3 cells and transfected cells using the TRIzol Reagent (Invitrogen; Thermo Fisher Scientific, Inc.), according to the manufacturer's instructions. For RT analysis, 2 μg of total RNA was reverse transcribed, using the Moloney Murine Leukemia Virus Reverse Transcriptase system (cat. no. 28025013; Thermo Fisher Scientific, Inc.). PCR was performed using Taq DNA polymerase system (cat. no. 10342020; Thermo Fisher Scientific, Inc.) by adding 2 μl RT reaction mixture in a final volume of 50 ul. DNA amplification conditions included an initial 5 min denaturation step at 94˚C and 35 cycles of 30 sec at 94˚C, 30 sec at 60˚C, and 60 sec at 72˚C. The PCR primers used were as follows: TRPM8 forward, 5'-GATTTT CACCAATGACCGCCG-3' and reverse, 5'-CCCCAGCAGCATTGATGTCG-3'; β-actin forward, 5'-AGAAGGATTCCTATGTGGGCG-3' and reverse, 5'-CATGTCGTCCCAGTTGGTGAC-3'.

Cell Counting kit-8 (CCK-8) assays. Cell growth and viability were measured using cell proliferation and cytotoxicity reagent CCK-8 (MedChemExpress, Cat. HY-K0301), according to the manufacturers' instructions (Roche Applied Science, Mannheim, Germany). The protocol was as follows: LNCaP or PC3 transfected and untransfected cells ($5x10^3$ per well) were cultured in a 96-well plate, ten wells per group. Following incubation in complete medium for 72 h, siRNA-transfection for 24 h or EPI-incubation for 48 h, fresh culture medium (which served as solvent for formazan generated from WST-8 by 1-Methoxy PMS) with 10 μl CCK-8 working solution was added and the mixture was incubated for another 4 h at 37˚C. For the examination of chemosensitivity, cells were incubated for 12 h to allow cells to attach to the plate surface, and then cells were treated with different concentrations (0,

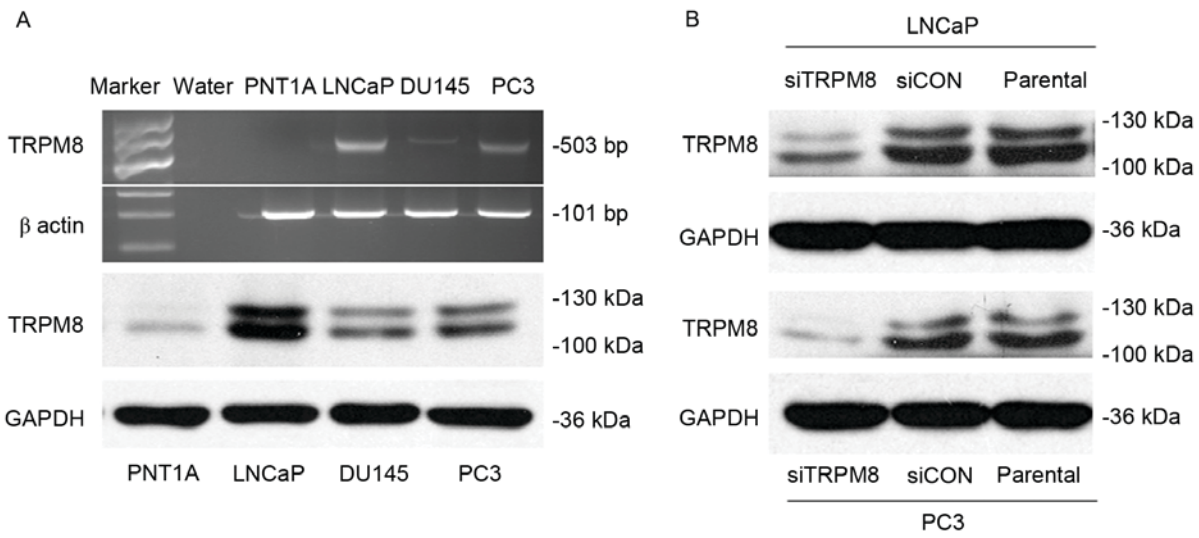

Figure 1. Expression of TRPM8 in cancerous and non-cancerous prostate cells and in siTRPM8 and siCON cells. (A) The expression of TRPM8 in PNT1A, LNCaP, DU145, and PC3 cells was detected by RT-PCR (above) and western blot (lower). β-actin and GAPDH were used as an internal standard in RT-PCR and western blot analysis, respectively. (B) The knockdown efficiency of siTRPM8 in LNCaP and PC3 cells. TRPM8 expression was evidently decreased in siTRPM8 cells compared with the parental and siCON cells. siTRPM8, small interfering RNA targeting transient receptor potential cation channel subfamily M member 8; CON, negative control.

200, 400, 600, 800 and 1,000 ng/ml respectively) of EPI for 72 h. To verify the role of MAPK signal pathway, p38 inhibitor (SB203580, 20 μM) (cat. no. S8307; Sigma-Aldrich; Merck KGaA,) and JNK inhibitor (SP600125; 10 μM; cat. no. S5567; Sigma-Aldrich; Merck KGaA) were added in the culture medium 2 h prior to the addition of EPI. The optical density at 490 nm was read using the enzyme-linked immunoassay reader. The cell viability index was calculated using the optical density (OD), according to the formula: Experimental OD value/control OD value x100. The experiments were repeated three times.

Western blot assay. The parental PNT1A, DU145, LNCaP and PC3 cells and transfected cells were washed twice with ice-cold PBS and solubilized in 1% Triton X-100 lysis buffer (cat. no. 9803; Cell Signaling Technology Inc., Danvers, MA, USA) on ice, then quantified using a BCA kit (cat. no. 23209; Thermo Fisher Scientific, Inc.). TRPM8 and GAPDH protein expression was assayed by western blotting using 10% SDS-PAGE and transferred to a polyvinylidene difluoride membrane (EMD Millipore, Billerica, MA, USA), with anti-TRPM8 antibodies (polyclonal, 1:500 in TBST), and anti-GAPDH specific antibodies (1:3,000 in TBST). Membranes were blocked by 5% skim milk solution at room temperature for 30 min and then incubated with primary antibodies against TRPM8 (1:500; cat. no. ab3243; Abcam, Cambridge, UK), p-JNK (1:1,000; cat. no. 4668P), total JNK (1:1,000; cat. no. 9258P), p-p38 (1:1,000; cat. no. 4511P) and total p38 (1:1,000; cat. no. 9212P; all Cell Signaling Technology, Inc.) and GAPDH (1:1,000; cat. no. sc-166574; Santa Cruz Biotechnology, Inc., Dallas, TX, USA) overnight at 4˚C, followed by incubation with a secondary antibody (cat. no. sc-2005; goat anti-mouse IgG-HRP, 1:2,000; Santa Cruz Biotechnology, Inc.) for 1 h at room temperature. The membrane was washed three times in TBST for 5 min each time. Immunoreactive proteins were detected using the SuperSignal West Pico Chemiluminescent Substrate Western Blot Detection system (cat. no. 34080; Thermo Fisher Scientific, Inc.) according to the protocol of the manufacturer. All experiments were repeated at least three times.

Flow cytometry analysis of apoptosis. The parental LNCaP and PC3 cells and transfected cells were incubated in a binding buffer (BD Pharmingen; BD Biosciences, San Diego, CA, USA) containing fluorescein isothiocyanate (FITC)-conjugated Annexin V (BD Pharmingen; BD Biosciences) and propidium iodide (BD Pharmingen; BD Biosciences, San Diego, CA, USA) at room temperature for 5 min in the dark. A flow cytometer (FACSCanto II, BD Biosciences, Franklin Lakes, NJ, USA) was used to detect the proportion of apoptotic cells and analyzed using FACSDiva version 6.1.3 (BD Biosciences).

Statistical analysis. All data is presented as the mean ± standard deviation. Statistical analysis was performed using SPSS for Windows, version 16.0 (SPSS, Inc., Chicago, IL, USA); $P<0.05$ was considered to indicate a statistically significant difference. Statistical analysis was conducted using the one-way analysis of variance followed by the SNK method.

Results

Expression of TRPM8 in different prostate cancer cell lines. Using RT-PCR, and western blot analysis, the expression of TRPM8 among different prostate cell lines, including human immortalized prostate epithelia cell line PNT1A, and three prostate cancer cell lines LNCaP, DU145, and PC3. The expression of TRPM8 mRNA and protein was higher in LNCaP and PC3 cells than in DU145; however, expression was almost undetectable in PNT1A cells (Fig. 1A). The efficiency of siRNA-mediated siTRPM8-knockdown was also investigated

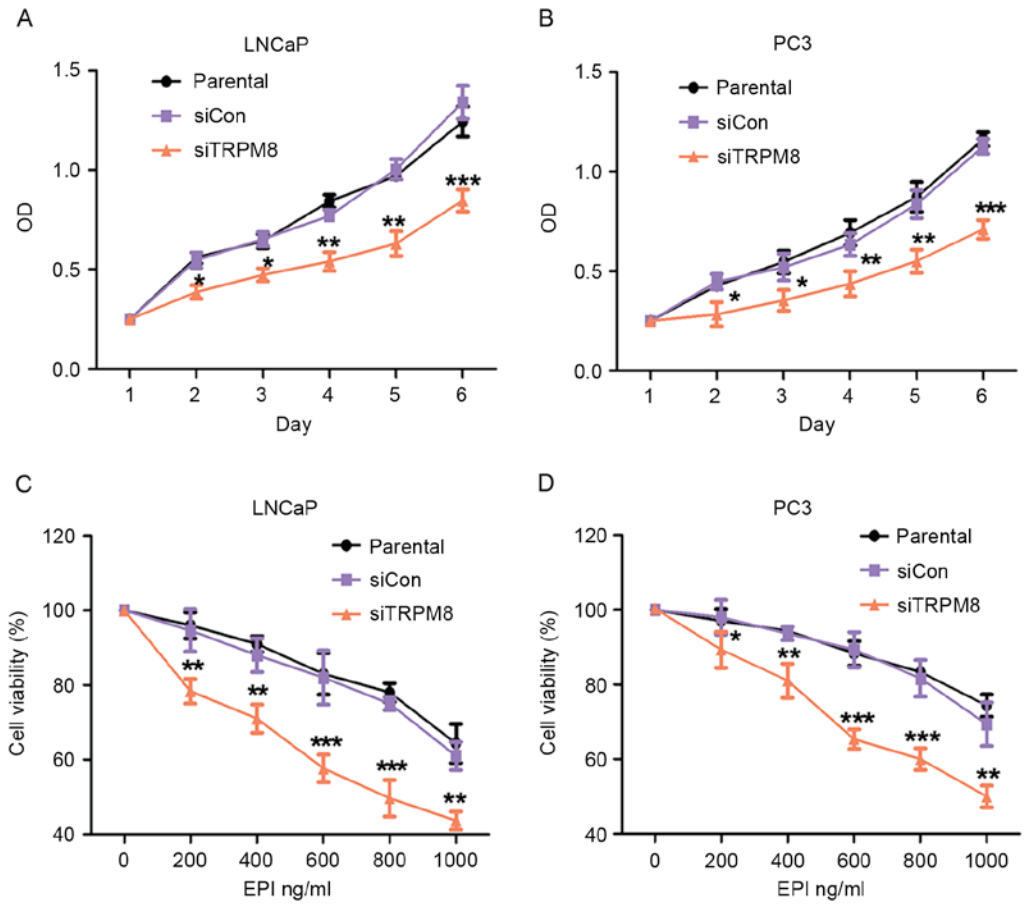

Figure 2. Knockdown of TRPM8 suppressed growth and enhanced EPI-induced growth inhibition of LNCaP and PC3 cells. (A and B) The effect of TRPM8-knockdown on (A) LNCaP and (B) PC3 cells proliferation was measured by CCK-8 assay. Growth of siTRPM8 cells was significantly suppressed at day 2 compared with parental and siCON cells. (C and D) Knockdown of TRPM8 significantly enhanced EPI-induced inhibition of viability of (C) LNCaP and (D) PC3 cells. Cells were incubated with vehicle (0.01% DMSO) or different concentrations (0, 200, 400, 600, 800, 1,000 ng/ml) of EPI for 48 h by CCK-8 assay, and are expressed as percentages relative to the control, which was taken as 100%, and treated with medium-containing vehicle (0.01% DMSO). *P<0.05, **P<0.01, ***P<0.001 vs. parental cells on indicated times or EPI concentrations. siTRPM8, small interfering RNA targeting transient receptor potential cation channel subfamily M member 8; EPI, epirubicin; CON, negative control; CCK-8, Cell Counting kit-8; DMSO, dimethyl sulfoxide.

by western blot analysis. Compared with parental and siCON cells, the expression of TRPM8 was markedly downregulated in the cells transfected with siTRPM8 in LNCaP and PC3 cells (Fig. 1B).

siTRPM8 inhibited cellular proliferation and enhanced EPI chemosensitivity in LNCaP and PC3 cells. The effect of TRPM8 knockdown on the proliferation of LNCaP and PC3 cells was assessed using CCK-8 assays. According to the results of CCK-8 assay, there was no statistical difference between parental and siCON cells, however the siTRPM8 cells grew more slowly than the parental and siCON cells and the difference was more pronounced after day 2 (Fig. 2A and B).

Next, the effect of siTRPM8 on chemosensitivity to EPI was assessed using a drug sensitivity test by CCK-8 assays. Compared with the parental and siCON cells, the viability of siTRPM8 cells was markedly weakened in a dose-dependent manner following incubation with EPI at the indicated concentration for 48 h (Fig. 2C and D). The viability of siTRPM8 cells was significantly lower than the parental and siCON cells when treated with 600 μM EPI, for LNCaP (siTRPM8, 58.37±2.14% vs. parental, 82.31±7.60%, P<0.001; siCON, 84.03±3.80% vs. parental cells, P>0.05) and PC3 (siTRPM8, 64.18±4.93% vs. parental cells, 85.34±2.17%, P<0.001; siCON, 85.42±2.40% vs. parental cells, P>0.05) cells (Fig. 2C and D).

siTRPM8 promoted EPI-induced apoptosis. When cells treated with siTRPM8 were compared with the parental and siCON cells, no significant change in the proportion of cells undergoing apoptosis was observed, as analyzed by flow cytometry (Fig. 3). Nevertheless, siTRPM8 facilitated cell apoptosis following treatment with EPI, which was confirmed by the Annexin V-FITC flow cytometry analysis. The concentration of EPI used in the following experiments was set at 600 ng/ml, according to the results of the CCK-8 assay (Fig. 3). This flow cytometry analysis revealed that knockdown of TRPM8 facilitated EPI-induced cell apoptosis when compared with the parental and siCON cells in LNCaP (siTRPM8, 72.28±3.34%; siCON, 50.84±1.37%; P<0.01) and PC3 (siTRPM8, 45.61±3.02%; siCON, 21.17±2.94%; P<0.05) cells (Fig. 3A and B).

Silencing TRPM8 activated the MAPK signal pathways. Whether MAPK pathways were involved in the action of

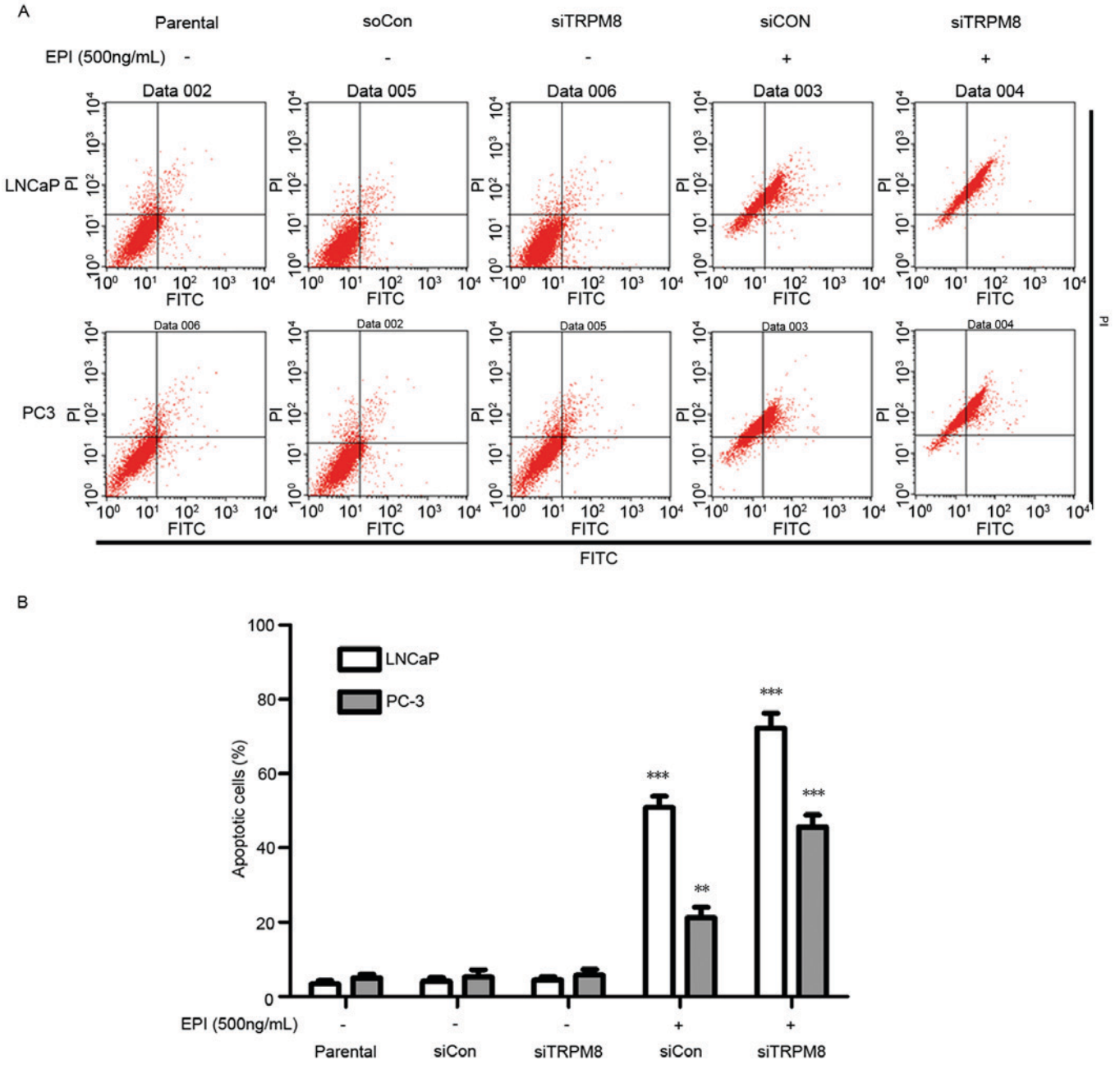

Figure 3. Knockdown of TRPM8 enhanced EPI-induced apoptosis, analyzed by flow cytometry. (A) LNCaP (above) and PC3 (below) cells were incubated with 500 ng/ml EPI for 48 h and then was harvested for apoptosis analysis. (B) The results of the flow cytometry analysis were quantified and expressed in histograms. The figures are representative of three experiments. **P<0.01, ***P<0.001, compared with the parental group. siTRPM8, small interfering RNA targeting transient receptor potential cation channel subfamily M member 8; EPI, epirubicin; CON, negative control.

siTRPM8 was also investigated. Levels of p-p38 and p-JNK increased in siTRPM8 cells, when compared with parental and siCON cells, with levels of total protein remaining unchanged (Fig. 4). To confirm the involvement of the MAPKs, the effects of the p38 inhibitor (SB203580, 20 μM) and JNK inhibitor (SP600125, 10 μM) on the EPI-mediated proliferative inhibition in siTRPM8 cells were analyzed by CCK-8 assays. These specific inhibitors of p38 and JNK attenuated the enhancement of EPI chemosensitivity of siTRPM8 (Fig. 5C). The efficiency of SB203580 and SP600125 were evaluated by western blot; the phosphorylation of p38 and JNK was evidently increased in siTRPM8 when compared with parental and siCON cells, thus lowering the apoptotic thresholds. Taken together, these results indicated that MAPK pathways at least partially participated in the role of EPI sensitization via siTRPM8 treatment in prostate cancer cells.

Discussion

TRPM8 is considered to be a novel prostate-specific marker; its expression is increase in PCa (2). Previous studies had indicated that TRPM8 expression increases only in early-stage PCa, decreasing markedly as tumor progresses to the late, invasive, androgen-independent stage (9,25). However, levels of TRPM8 expression in PC3 cells have been disputed (26,27). Using the nested RT-PCR method Kim *et al* (28) verified

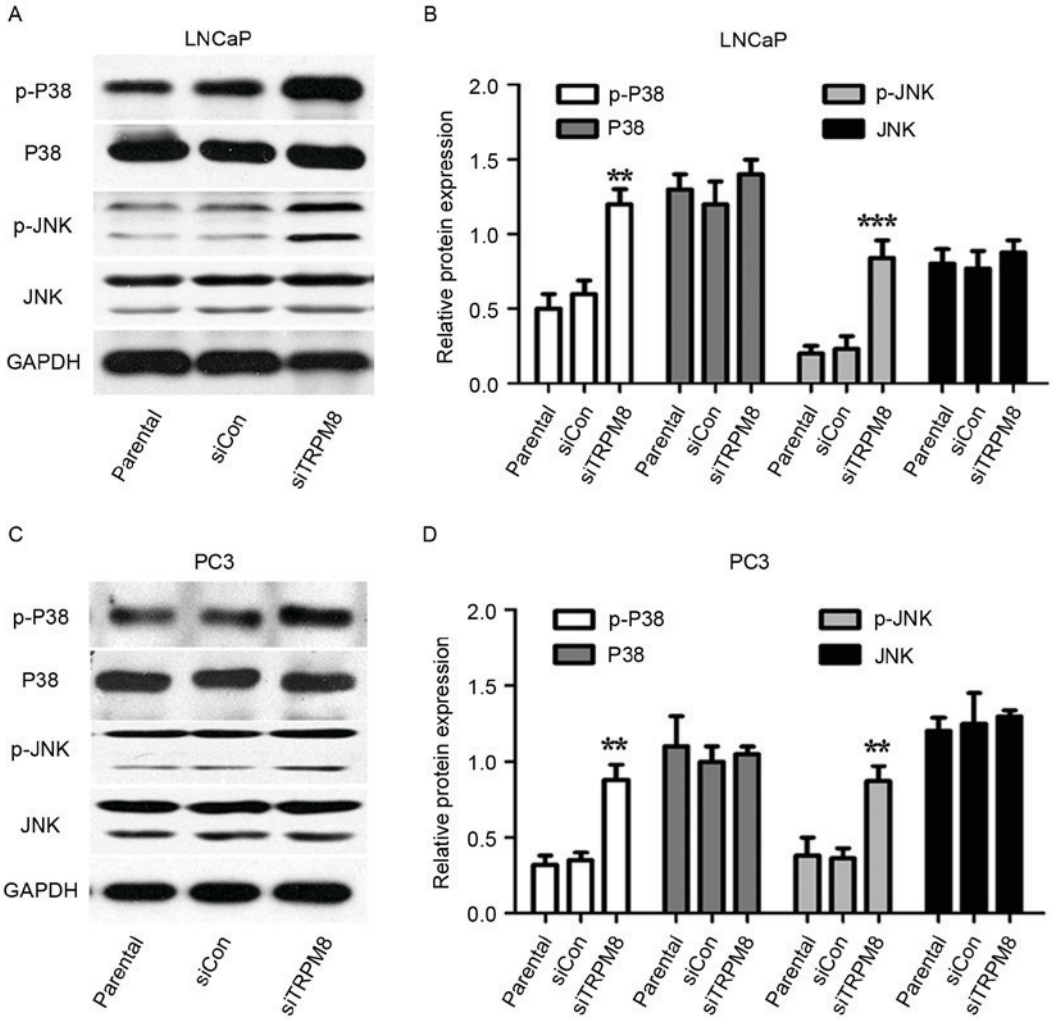

Figure 4. Mitogen-activated protein kinase signal pathways may partially involve in the sensitization activity of siTRPM8 towards LNCaP and PC3 cells. (A-D) Western blot analysis in (A) LNCaP cells, with (B) quantification and in (C) PC3 cells, with (D) quantification. This analysis was performed performed to investigate the expression of p-p38, p38, p-JNK, and JNK in parental, siCON, siTRPM8 cells. **P<0.01, ***P<0.001, compared with the parental group. p-p38, phosphorylated p38 mitogen-activated protein kinase; JNK, c-Jun N-terminal kinase; siTRPM8, small interfering RNA targeting transient receptor potential cation channel subfamily M member 8; CON, negative control.

that the expression of TRPM8 in LNCaP and PC3 cells was comparable. The present study investigated the expression of TRPM8 in cancerous and non-cancerous prostate epithelial cells by RT-PCR and western blotting (TRPM8 expression levels: LNCaP>PC3>DU145>PNT1A).

A previous study revealed that BCTC, a potent and representative antagonist of TRPM8, exerts an antitumor effect on androgen-independent prostate cancer DU145 cells by p-AKT, cyclin D1, CDK2 and CDK6, p-GSK-3β and also MAPK signal pathways (23). Zhang *et al* (2) and Valero *et al* (17) also demonstrated the antitumor effect of knockdown or blockade of TRPM8 in PCa cells. Valero *et al* (17) provided evidence that the knockdown and antagonism (including by BCTC) of TRPM8 could inhibit the proliferation, cell cycle progression and migration of PCa cells. However, whether targeting TRPM8 can influence the chemosensitivity of prostate cancer remains unknown. Therefore, the present study uncovered the possible influence of TRPM8-knockdown on the chemosensitivity of prostate cancer and the precise mechanism involved.

The data produced by the present study revealed that the RNA interference-mediated depletion of TRPM8 evidently inhibited the proliferation of LNCaP and PC3 cells. Although siTRPM8 treatment failed to induce apoptosis in prostate cancer cells alone, it facilitated cell apoptosis when EPI was administered, which indicated that the chemosensitivity of LNCaP and PC3 cells was enhanced by TRPM8-knockdown. This result revealed that EPI, which is routinely used as a therapeutic for treatment of late-stage prostate cancer but to which resistance is readily developed, could be used in combination with knockdown of TRPM8.

MAPK family members are known to control cell cycle progression at various stages in cell type- and context-specific manners. The present study found that levels of p-p38 and p-JNK increased in siTRPM8-LNCaP and siTRPM8-PC3 cells, compared with parental and siCON cells. Furthermore, specific inhibitors of p38 and JNK attenuated the enhancement of EPI chemosensitivity induced by siTRPM8, which indicated that MAPK pathways are partially involved in the sensitization activity of siTRPM8 towards LNCaP and PC3 cells.

In summary, the present study demonstrates that the knockdown of TRPM8 using a specific siRNA reduced the

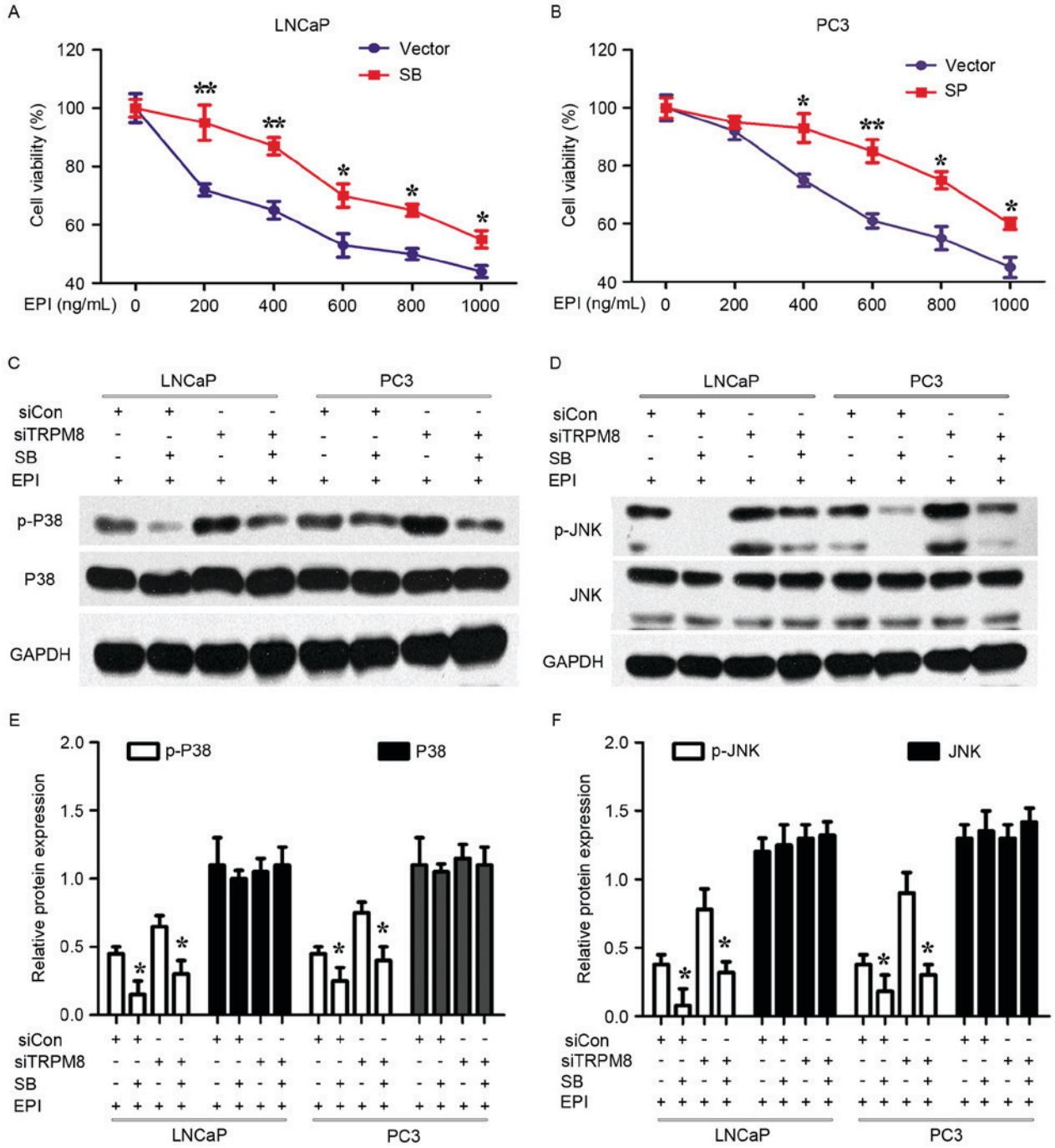

Figure 5. Specific inhibitors of p38 and JNK attenuated the enhancement of EPI chemosensitivity of siTRPM8. (A and B) siTRPM8 Cells were treated with the p38 inhibitor SB203580 (20 μM) and JNK inhibitor SP600125 (10 μM) for 4 h, respectively, and then treated with 500 ng/ml EPI in (A) LNCaP and (B) PC3 cells. After 48 h treatment, cell viability was determined by Cell Counting kit-8 assay. (C and D) Alteration of mitogen-activated protein kinase signal pathways when (C) LNCaP and (D) PC3 cells were treated with p38 and JNK inhibitors. siTRPM8 increased the expression of p-P38 and p-JNK, which lowered the threshold of EPI-induced apoptosis. (E and F) Quantification of the results of (C and D), expressed in histograms. *P<0.05, **P<0.01, compared with cells treated with the vehicle. p-p38, phosphorylated p38 mitogen-activated protein kinase; JNK, c-Jun N-terminal kinase; siTRPM8, small interfering RNA targeting transient receptor potential cation channel subfamily M member 8; CON, negative control; EPI, epirubicin.

proliferative ability and enhanced the chemosensitivity of prostate cancer LNCaP and PC3 cells. This finding is partially attributed to the alteration of the MAPK signal pathways. These results reveal that RNA interference-mediated depletion of TRPM8 is a therapeutic strategy with substantial potential for treating prostate cancer, including CRPC, which can provide novel insights into the understanding of prostate cancer biology.

Acknowledgements

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