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Reciprocal positive regulation between TRPV6 and NUMB in PTEN-deficient prostate cancer cells

Sung-Young Kim [a,b,1], Chansik Hong [a,b,1], Jinhong Wie [a,b], Euiyong Kim [c], Byung Joo Kim [d], Kotdaji Ha [a,b], Nam-Hyuk Cho [b], In-Gyu Kim [b], Ju-Hong Jeon [a,b], Insuk So [a,b,*]

[a] Department of Physiology, Seoul National University College of Medicine, Seoul 110-799, Republic of Korea
[b] Department of Biomedical Sciences, Seoul National University College of Medicine, Seoul 110-799, Republic of Korea
[c] Department of Physiology, College of Medicine, Inje University, Busan 614-735, Republic of Korea
[d] Division of Longevity and Biofunctional Medicine, Pusan National University School of Korean Medicine, Yangsan 626-870, Republic of Korea

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ABSTRACT

Calcium acts as a second messenger and plays a crucial role in signaling pathways involved in cell proliferation. Recently, calcium channels related to calcium influx into the cytosol of epithelial cells have attracted attention as a cancer therapy target. Of these calcium channels, TRPV6 is overexpressed in prostate cancer and is considered an important molecule in the process of metastasis. However, its exact role and mechanism is unclear.

NUMB, well-known tumor suppressor gene, is a novel interacting partner of TRPV6. We show that NUMB and TRPV6 have a reciprocal positive regulatory relationship in PC-3 cells. We repeated this experiment in two other prostate cancer cell lines, DU145 and LNCaP. Interestingly, there were no significant changes in TRPV6 expression following NUMB knockdown in DU145. We revealed that the presence or absence of PTEN was the cause of NUMB–TRPV6 function. Loss of PTEN caused a positive correlation of TRPV6–NUMB expression. Collectively, we determined that PTEN is a novel interacting partner of TRPV6 and NUMB. These results demonstrated a novel relationship of NUMB–TRPV6 in prostate cancer cells, and show that PTEN is a novel regulator of this complex.

1. Introduction

Changes in intracellular free calcium (Ca^{2+}) play a major role in many cellular processes, and deregulation of Ca^{2+} signaling is a feature of a variety of diseases [1,2]. Cancer cells use the same Ca^{2+} channels, pumps, and exchangers as non-malignant cells. However, there are often key alterations in Ca^{2+} channels and pumps in cancer cells. Such changes in cancer cells may include the expression of Ca^{2+} channels or pumps that are not normally present in non-malignant cells of the same cell type, pronounced changes in the level of expression, altered cellular localization, altered activity through changes in post-translational modification, gene mutations, and changes in activity or expression associated with specific cancer-relevant processes [3–6].

Prostate cancer is the most common type of cancer and the second leading cause of cancer death in men in the United States [7]. The growth of prostate epithelial cells requires androgen. Since the growth of prostate cancer cells depends on the presence of androgens, androgen deprivation therapy has been the primary treatment for patients with metastatic prostate cancer. Hormone-refractory prostate cancer is the result of regrowth of prostate cancer cells that have adapted to the hormone-deprived environment of the prostate. The complex mechanism of hormone resistance has been the topic of research in many laboratories that have analyzed the process from different angles [8–12]. Advances in genomic research have recently identified various molecular features with importance for prostate cancer biology [13,14]. There have been many studies of the molecular factors that could mediate an increase in the risk of prostate cancer progression to therapy resistance. One recently implicated molecular factor is PTEN, a protein/lipid phosphatase. The main mechanism of tumor suppression by PTEN is the maintenance of cellular PIP-3 at low levels, thus inhibiting the PI3K–AKT pathway and contributing to cellular apoptosis or cell cycle arrest [15–17]. PTEN has been found to be inactivated in a wide variety of human cancers and cancer cell lines, including prostate cancer. The loss of one copy of the *PTEN* gene contributes to prostate tumor initiation, while further reduction in PTEN

* Corresponding author at: Department of Physiology, Seoul National University College of Medicine, Seoul 110-799, Republic of Korea. Fax: +82 2 763 9667.
E-mail address: insuk@snu.ac.kr (I. So).
[1] These authors contributed equally to this work.

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expression supports the invasion and metastatic behavior of prostate cancer [18–22].

Transient receptor potential (TRP) channels are superfamily of cation channel proteins that are expressed in many organs. On the basis of structural homology, the superfamily can be subdivided into seven main subfamilies: TRPC (canonical), TRPV (vanilloid), TRPM (melastatin), TRPP (polycystin), TRPML (mucolipin), TRPA (ankyrin) and TRPN (no mechanoreceptor potential C, NOMPC) [23,24]. Numerous functional studies showed that TRPs are involved in different cancer cells [25–27]. Among them, TRPV6 is reported to play a role in prostate cancer, where it is considered as a diagnostic and prognosis marker. In healthy and benign human prostate tissue, the expression of TRPV6 mRNA is either low or undetectable. However, when the Gleeson score is high, the degree of metastasis outside the prostate displays substantial expression of TRPV6 mRNA [28,29]. Increased expression of TRPV6 mRNA is also observed in human prostate cancer cell lines (LNCaP, PC-3) compared to normal and benign epithelial cells (PrEC, BPH1) [30].

In our previous study, we showed that NUMB, a tumor suppressor gene, inhibits the activity of TRPV6 via electrostatic interaction in breast cancer cells [31]. NUMB protein is responsible for various signal transduction pathways (Hedgehog, P53), endocytosis (cargo internalization and recycling), cell polarity determination, and ubiquitination [32]. In cancer, NUMB functions as a tumor suppressor via the stabilization of P53 [33]. NUMB forms a tricomplex with P53 and its negative regulator, MDM2. Loss of NUMB expression has been observed in mammary and lung cancer but there has been no study to date regarding their potential roles and relationships in prostate cancer [34,35]. In this report, we investigated the role of the NUMB–TRPV6 interaction in prostate cancer cells.

2. Materials and methods

2.1. Materials

Anti-c-myc (sc-40) and anti-GFP (sc-8334) were obtained from Santa Cruz Biotechnology, Inc. The anti-TRPV6 (ACC-036) antibody was obtained from Alomone Labs.

2.2. Cell culture and transfection

PC-3, Du145 and LNCaP were cultured in RPMI (Hyclone), HEK293 were cultured in DMEM (Hyclone) supplemented with 10% heat-inactivated FBS and penicillin (100 U/ml), streptomycin (100 μg/ml) at 37 °C in a 5% CO_2 humidified incubator. The transfection was performed with Fugene-6 according to the manufacturer's instructions.

2.3. DNA plasmids

DNA plasmids cloning were performed as described previously [31].

2.4. Co-immunoprecipitation

HEK293 cells transfected with the indicated plasmids were lysed in ice-cold lysis buffer (50 mM Tris–Cl (pH 7.4), 1 mM EDTA, 150 mM NaCl, 0.5% Triton X-100 protease inhibitor and phosphatase inhibitor (Calbiochem)). The soluble fractions of cell lysates were isolated by centrifugation at 15,000*g* for 10 min. For immunoprecipitations, primary antibodies were added to the lysates and incubated with rotation for 1.5 h at 4 °C. 25 μl of 50% slurry of protein G-agarose was then added, and the incubation continued for an additional 1.5 h. Immunoprecipitates were washed four times with lysis buffer containing 0.2% Triton X-100. Immunoprecipitated proteins were denatured by the addition of 30 μl of 2× sample buffer and boiling for 1 min, resolved by SDS–PAGE, and analyzed by immunoblotting as described.

2.5. Calcium influx assay

The experiments were performed in an NT solution containing 3.6 mM KCl, 10 mM HEPES, 1 mM $MgCl_2$, 145 mM NaCl, 2 mM $CaCl_2$, 5 mM Glucose, pH 7.4. The ratiometric measurement of $[Ca^{2+}]I$ was performed using Fura-2 AM (molecular probe). The cells were grown in 24-well dishes and loaded with 5 μM of Fura-2 AM for 30 min at 37 °C. The Fura-2 fluorescence was measured at a 510 nm emission with a 340/380 nm dual excitation using a DG-4 illuminator.

2.6. Statistics

The data were compared by using ANOVA followed by a post hoc test. The values are given as the means + SEM. The results were considered to be significantly different when $P < 0.05$.

3. Results

3.1. Reciprocal positive regulation of TRPV6 and NUMB

Knockdown of NUMB in PC-3 cells resulted in a substantial decrease in the amount of TRPV6 protein (Fig. 1A). These results contrast with our previous results from MCF-7 human breast cancer cells, in which the protein levels of TRPV6 were elevated following knockdown of NUMB. We also showed that TRPV6 and NUMB undergo reciprocal regulation. To confirm these data, we used TNFα, a cytokine that decreases the expression of NUMB [39]. In our experiments, TNFα also reduced TRPV6 as well as NUMB (Fig. 1B). Furthermore, NUMB levels were significantly decreased in siTRPV6-expressing PC-3 cells (Fig. 1C). Because Ca^{2+} reduces the expression of TRPV6 [40], we tested the levels of NUMB after dose-dependent treatment with Ca^{2+} (Fig. 1D). According to our predictions, both TRPV6 and NUMB expression decreased. β-Glucuronidase or β-ionone results in an increase in the amount of TRPV6 [41,42]; interestingly, activation of TRPV6 with β-glucuronidase or β-ionone resulted in increased expression of NUMB in PC-3 cells (Fig. 1E and F), indicating that NUMB expression is positively regulated by TRPV6. Taken together, we identified reciprocal positive regulation between NUMB and TRPV6.

3.2. NUMB has a selective effect on TRPV6 depending on the prostate cancer cell line

To determine whether NUMB–TRPV6 protein levels are correlated in other human prostate cancer cell lines, we evaluated two cell lines, DU145 (androgen-refractory prostate cancer cells similar to PC-3) and LNCaP (androgen-responsive prostate cancer cells). As shown in Fig. 2A, when expression of NUMB was decreased, the level of TRPV6 protein also decreased in LNCaP cells. Interestingly however, there was no change in DU145 cells (Fig. 2B). The results of the calcium influx assay reflected this observation (Fig. 2C and D). This phenomenon and the difference between DU145 and other cells may be due to genetic mutations related to metastasis. Numerous studies have implicated the loss of PTEN in prostate cancer progression [18–22]; PC-3 and LNCaP cells do not express PTEN, a potent tumor suppressor gene, in comparison to DU145 cells that do have PTEN.

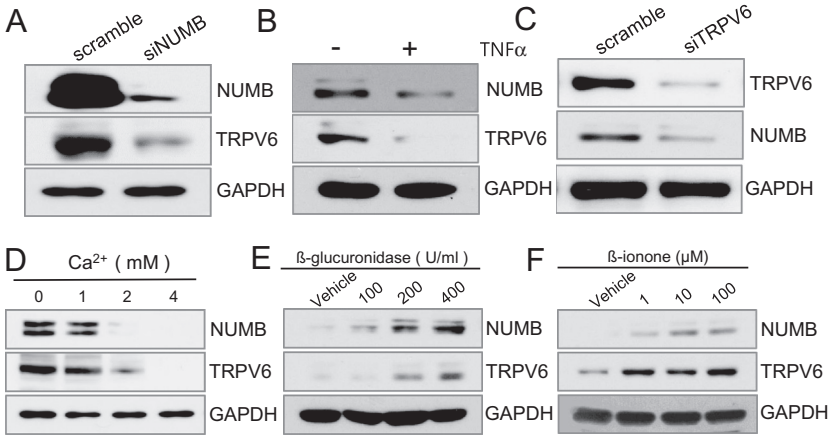

Fig. 1. Role of NUMB and TRPV6 in PC-3 cells. (A) Effect of NUMB knockdown in TRPV6 expression. PC-3 cells were transfected with siNUMB and scramble siRNA for 72 h. Cell lysates were analyzed by Western blotting. (B) Effect of TNFα in NUMB and TRPV6 expression. PC-3 cells were treated with TNFα (20 ng/μl) for 24 h and analyzed by Western blotting. (C) siTRPV6 and scramble siRNA were transfected in PC-3 cells. After 72 h, Western blotting was used to detect the expression of TRPV6 and NUMB. (D–F) PC-3 cells were exposed to various concentrations of Ca^{2+}, β-glucuronidase, β-ionone for 24 h and then analyzed by Western blotting.

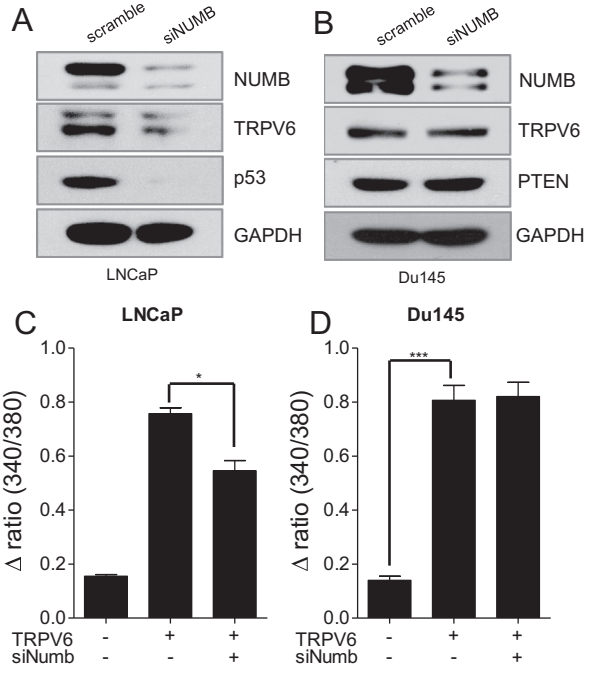

Fig. 2. Effect of siNUMB on Du145 and LNCaP prostate cancer cells. (A, B) Western blotting analysis of TRPV6 expression after transfection of siNUMB and scramble siRNA for 72 h. (C, D) Du145 and LNCaP cells were transfected with pEYFP-C1 TRPV6 and siNUMB during 72 h and calcium influx assay was performed.

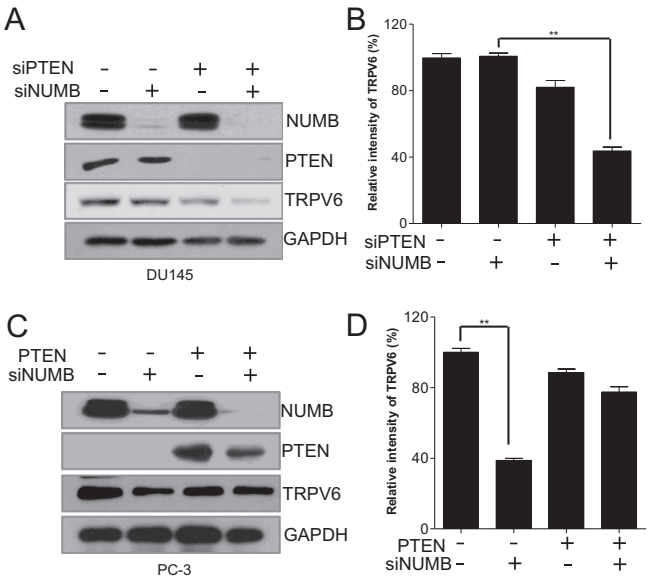

Fig. 3. Effect of PTEN on PC-3 and Du145 cells. (A) Du145 cells were transfected with siPTEN and/or siNUMB for 72 h and then Western blotting was performed. (B) The relative TRPV6 to GAPDH ratio was plotted based on quantification of the band intensities ($n = 3$). (C) PC-3 cells were transfected with PTEN plasmid and/or siNUMB for 72 h and then Western blotting was performed. (D) The relative TRPV6 to GAPDH ratio was plotted based on quantification of the band intensities ($n = 3$).

3.3. PTEN influences the regulation of TRPV6 by NUMB

Next, we examined whether knockdown of PTEN would result in the alteration in TRPV6 protein expression. Transfection of DU145 cells with siPTEN and siNUMB led to significantly decreased levels of TRPV6 protein compared with knockdown of each protein (Fig. 3A and B). PTEN overexpression in PC-3 cells reversed the reduced expression of TRPV6 by the knockdown of NUMB (Fig. 3C and D). These results suggest PTEN loss was responsible for the positive correlation between NUMB and TRPV6. However, overexpression of PTEN could not increase the expression of TRPV6 similar to that observed in MCF-7 cells when NUMB was knocked down in PC-3 cells.

3.4. PTEN complexes with NUMB and TRPV6

To study the possibility of a molecular association of PTEN with NUMB–TRPV6 complexes, we performed co-immunoprecipitation experiments. As shown in Fig. 4A and B, PTEN interacted with NUMB and TRPV6. We confirmed these interactions endogenously (Fig. 4C and D). Taken together, we identified that PTEN was a novel binding partner of the NUMB and TRPV6 complex.

4. Discussion

We showed that knockdown of NUMB reduced expression of TRPV6 protein. Additionally, there was a significant positive correlation between TRPV6 and NUMB in PC-3 cells. The positive

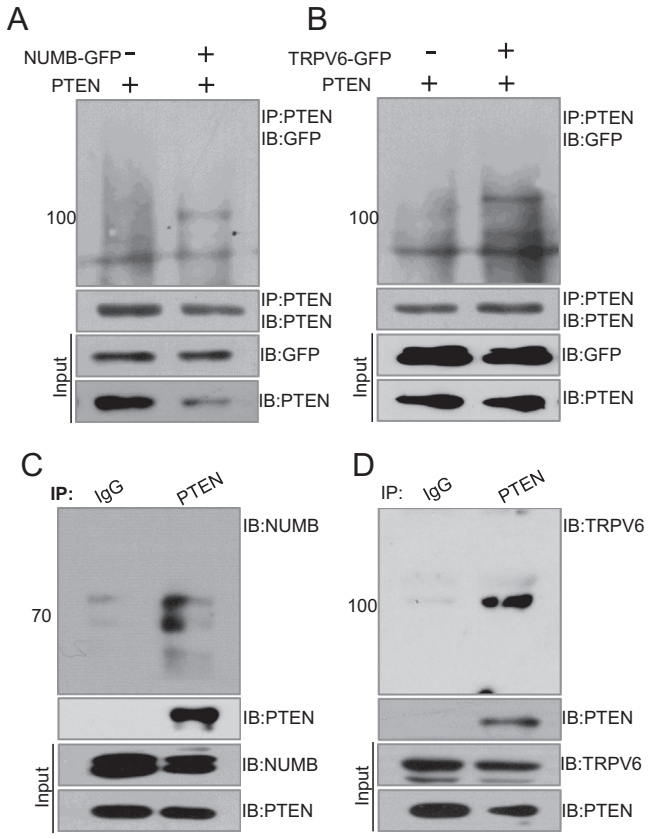

Fig. 4. Interaction of TRPV6–NUMB–PTEN. (A) NUMB-GFP and PTEN plasmids were transfected in HEK293 cells. After 24 h, Co-IP was performed. (B) TRPV6-GFP and PTEN plasmids were transfected in HEK293 cells. After 24 h, Co-IP was performed. (C, D) Co-immunoprecipitation of PTEN, NUMB, TRPV6. Whole cell lysates from Du145 cells were prepared, and immunoprecipitation was performed with anti-PTEN followed by immunoblotting with antibodies against Numb and TRPV6.

correlation with TRPV6 contrasted with our predicted findings due to the known function of NUMB as a tumor suppressor gene. In a previous study, we identified that NUMB and TRPV6 undergo reciprocally negative regulation via electrostatic interaction in MCF-7 breast cancer cells. These results indicate that the function of NUMB toward TRPV6 is cancer type-selective. Another prostate cancer cell line, LNCaP, showed similar results. However, DU145 cells hardly displayed any change in TRPV6 when NUMB was knocked down. This clearly demonstrates that NUMB could not inhibit the activity of TRPV6 in prostate cancer cell lines. From these findings we have two questions. First, why could NUMB not function as a tumor suppressor? NUMB has recently been found to play an important role in cancers, such as medulloblastoma, and breast, lung and salivary gland carcinomas. However, the function of NUMB in prostate cancer has yet to be elucidated. It is hypothesized that the regulatory factors in NUMB failed to function in prostate cancer due to unknown mutations.

Second, why do DU145 cells behave differently to other prostate cancer cell lines? We demonstrated that PTEN is involved in the aforementioned distinction. When screening advanced metastatic prostate cancer cell lines for PTEN, we observed a loss of expression in PC-3 and LNCaP cells whereas DU145 cells expressed WT PTEN. All three cell lines were positive for TRPV6 and NUMB expression. We investigated PTEN in null PC-3 and LNCaP and WT DU145 prostate cancer cell lines to characterize the involvement of PTEN in NUMB-mediated functions of TRPV6. Knockdown of PTEN in DU145 cells displayed similar results as PC-3 cells, as well as those resulting from transfection of PTEN in PC-3 cells. The recent surge of high-throughput sequencing of cancer genomes has supported an expanding molecular classification of prostate cancer. Multiple studies confirmed the functional involvement of PTEN in invasion and metastasis. It was reported that loss or mutation of PTEN occurred in 30% of primary prostate cancer and in 63% of metastatic disease. PTEN gene mutations and loss of expression were frequently linked to advanced stages and high Gleason score of prostate carcinoma in xenografts and paraffin-embedded tissues. It has been reported that PTEN mutations have an influence on the function of significant genes. Our results indicate that increased expression of TRPV6 is strongly related to loss of PTEN as prostate cancer progresses.

In conclusion, determination of the relationship between PTEN, NUMB and TRPV6 may lead to new treatment options, especially for aggressive, androgen-insensitive cancers that express low or diminished levels of PTEN and high levels of TRPV6.

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