

# Suppression of STIM1 inhibits the migration and invasion of human prostate cancer cells and is associated with PI3K/Akt signaling inactivation

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**Abstract.** Store-operated calcium entry (SOCE) plays an important role in the invasion and migration of cancer cells. Stromal-interacting molecule 1 (STIM1) is a critical component in the SOCE. STIM1 has been attracting more and more attention due to its oncogenic potential. STIM1 inhibition suppresses cell proliferation, migration and invasion in a variety of cancer models both *in vitro* and *in vivo*. However, the role of STIM1 in prostate carcinogenesis, in particular, in tumor migration and invasion is unclear. Herein, we downregulated STIM1 in prostate cancer cells by lentivirus-mediated short hairpin (shRNA), and then studied its impacts on cell migration and invasion. We found that migration and invasion of prostate cancer cells were significantly inhibited after the suppression of STIM1. Furthermore, we demonstrated that the PI3K/Akt signaling pathway was inactivated by STIM1 knockdown. The PI3K inhibitor LY294002 synergized with STIM1 knockdown to inhibit cell motility. Our results revealed that STIM1 may act as a novel regulator to promote migration and invasion of prostate cancer cells and is associated with the activation of the PI3K/Akt signaling pathway.

## Introduction

Prostate cancer (PCa) is the most commonly diagnosed malignancy and the second leading cause of cancer-related deaths in the western male population, while metastasis contributes to

majority of deaths (1,2). The five-year survival rate of patients who present with localized disease is ~90%, which was significantly higher than those with metastatic disease. Only ~33% of men presented with metastatic tumors live beyond 5 years. Although prostate cancer treatment has achieved numerous breakthroughs in the last decade, effective therapies to prevent prostate cancer metastasis to other parts of body are still lacking (3). Therefore, novel approaches are urgently needed for the prevention and treatment of the metastasis of prostate carcinoma.

Calcium (Ca<sup>2+</sup>), an intracellular messenger, is indispensable for various cellular functions, such as proliferation, apoptosis and metastasis (4,5). In non-excitabile cells, store-operated calcium entry (SOCE) is the major influx of Ca<sup>2+</sup> and is regulated by a store-operated calcium channel (SOC), which consists of two important components, stromal-interacting molecule 1 (STIM1) and calcium release-activated calcium channel protein 1 (ORAI1). STIM1 is located in the endoplasmic reticulum (ER) and acts as Ca<sup>2+</sup> sensor. Once the Ca<sup>2+</sup> store in ER is depleted, STIM1 is activated and forms an oligomer with ORAI1, a transmembrane protein, to form pores for Ca<sup>2+</sup> influx (6).

STIM1 participates in a variety of cellular functions, including muscle contraction, the release of neurotransmitters and hormones, gene transcription, cell proliferation and metastasis (7). Recently, STIM1 has attracted more and more attention due to its oncogenic potential. STIM1 inhibition suppresses cell proliferation, migration and invasion in a variety of cancer models both *in vitro* and *in vivo* (8-11). However, to date, its function on metastasis of prostate cancer is unclear.

The phosphatidylinositol 3-kinase/protein kinase-B (PI3K/Akt) signaling pathway plays a pivotal role in cell growth, differentiation, proliferation and metastasis. PI3K phosphorylates and activates Akt, affecting its downstream signaling molecules. It has been demonstrated that activation of Akt promotes migration of tumor cells (12-14). Numerous studies have revealed that the PI3K/Akt signaling pathway is extensively activated in migration and invasion of

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various types of cancers, including liver, breast and pancreatic cancer (15-17). PI3K/Akt inhibition by specific inhibitors suppresses cell migration and invasion (18-21). Whether the PI3K/Akt signaling pathway is involved in the regulation of invasion and migration by STIM1 is unclear and needs to be further explored.

In the present study, we determined whether STIM1 knockdown inhibited cell migration and invasion of prostate cancer and further explored the potential underlying mechanism of STIM1 in the process with a focus on the regulation of the PI3K/Akt signaling pathway.

## Materials and methods

**Reagents.** The PI3K inhibitor LY294002 was obtained from Selleckchem (Houston, TX, USA). Matrigel was purchased from BD Biosciences (San Jose, CA, USA), Transwell Minicells were obtained from Millipore (Darmstadt, Germany). A BCA protein qualification kit was purchased from Pierce (Rockford, IL, USA). The anti-STIM1 antibody for immunohistochemistry (IHC) staining was purchased from Abgent (San Diego, CA, USA). Anti-STIM1 (4916), anti-p-Akt (Thr308) (13038), anti-t-Akt (4691), anti-GAPDH (5174), anti-E-cadherin (3195), anti-N-cadherin (13116), anti-vimentin (5741), anti-Snail (3879) and secondary antibodies anti-rabbit (14708) and anti-mouse (14709) antibodies were obtained from Cell Signaling Technology (Boston, MA, USA).

**Prostate tissue acquisition and IHC staining.** Prostate cancer specimens were obtained from prostate cancer patients undergoing prostatectomy or prostate biopsy. Benign prostatic hyperplasia (BPH) tissues were obtained from BPH patients undergoing surgery at the Second Affiliated Hospital of Soochow University and were used as a normal control. The present study was approved by the Research Ethics Committee of the Second Affiliated Hospital of Soochow University and was in compliance with the Helsinki Declaration. Tissues were examined by pathologists to confirm the diagnosis before IHC analyses. All specimens were fixed in 10% formalin, embedded in paraffin, and cut into 4- $\mu$ m thick slides. The slides were dewaxed, and the endogenous peroxidase activity was blocked by treatment with 3% hydrogen peroxide solution in methanol for 20 min. Non-specific binding was prevented by blocking with normal goat serum (1:100) for 30 min. The staining procedure was carried out using the avidin-biotin-peroxidase complex method. The expression of STIM1 was evaluated by staining with a mouse anti-STIM1 antibody. After incubation with a primary antibody for 60 min, the slides were washed using phosphate-buffered saline (PBS) 3 times, and then incubated with a biotinylated goat anti-mouse IgG (H+L) at 37°C for 30 min, followed by incubation with a streptavidin-biotinylated HRP complex (Sigma, St. Louis, MO, USA) for 30 min. Reactive products were visualized with 3,3'-diaminobenzidine (DAB) as the chromogen, and slides were counterstained with hematoxylin. Sections previously known to express STIM1 were included in each run, receiving either the primary antibody as the positive control or a mouse IgG as the negative control. Stained slides were observed by microscope.

All slides were evaluated twice at different time-points by two independent pathologists. The expression level of STIM1

was assigned a score based on the percentage of positive tumor cells over total tumor cells and their staining intensities. The proportion of positive tumor cells  $\leq 10\%$ , 11-25%, 26-50% and  $>50\%$  were scored as 1, 2, 3 and 4. In addition, when the staining intensities were a non-significant brown, a slight brown, a moderate brown and a deep brown, they were scored as 1, 2, 3 and 4. Then, the two scores were added. A score of 2-3 was graded as weak, and a score of 4-8 was graded as strong.

**Cell culture.** Normal prostate cell line RWPE-1 and prostate cancer cell lines LNCap, PC-3 and DU-145 were purchased from the American Type Culture Collection (ATCC; Manassas, VA, USA). These were cultured according to the instructions of ATCC. C4-2 was obtained from UroCor Inc., (Oklahoma City, OK, USA) and grown in RPMI-1640 medium containing 10% fetal bovine serum (FBS) (Atlanta Biologicals, Flowery Branch, GA, USA) and 1% penicillin-streptomycin solution (Invitrogen, Carlsbad, CA, USA). The cells were observed and images were obtained using an inverted microscope (Olympus, Tokyo, Japan). When the cell density reached 90-100%, the cells were sub-cultured and seeded to plates according to the experimental design. The culture medium was replaced by fresh one every 2-3 days or according to the experimental design.

**Total RNA isolation, cDNA reversion and polymerase chain reaction (PCR).** Total RNA was isolated using Trizol reagent (Invitrogen) according to the manufacturer's instructions. The concentration of total RNA was detected by UV spectrophotometry. RT-PCR was performed by the two-step method. Synthesis of cDNA was performed using a cDNA Synthesis kit (Thermo Fisher Scientific, Franklin Lakes, NJ, USA). The PCR reaction conditions were: 95°C for 5 min, 94°C for 30 sec, 56°C for 30 sec, 72°C for 30 sec for 40 cycles; the total volume was 20  $\mu$ l. GAPDH was used as an internal standard. The sequences of the primers used were: GAPDH forward, TGTGG GCATCAATGGATTTGG and reverse, ACACCATGTATTC CGGGTCAAT; and STIM1 forward, AGTCACAGTGAGAA GGCG AC and reverse, ACACCATGTATTCCGGGTCAAT. All experiments were performed in triplicate.

**Western blot analysis.** Total protein was extracted by radio immunoprecipitation assay (RIPA) buffer (0.15 mM NaCl, 0.05 mM Tris-HCl, pH 7.5, 1% Triton, 0.1% SDS, 0.1% sodium deoxycholate and 1% NP40). Sample extracts (30  $\mu$ g) were loaded to 12% SDS-polyacrylamide gels (PAGE) using a minigel apparatus and transferred to polyvinylidene difluoride (PVDF) membranes (both from Bio-Rad, Hercules, CA, USA). The membranes were blocked for 1 h with 5% skim milk, then incubated overnight with primary antibody. Then the membranes were washed using Tris-buffered saline with Tween-20 (TBST) 3 times and incubated with a secondary antibody for 1 h at room temperature. Blots were visualized by enhanced chemiluminescence (ECL) system.

**Construction and infection of lentivirus-mediated short hairpin vector.** For short hairpin RNA (shRNA)-mediated knockdown of STIM1, cells were transfected with lentiviral particles produced using the STIM1-pGCSIL-GFP plasmid

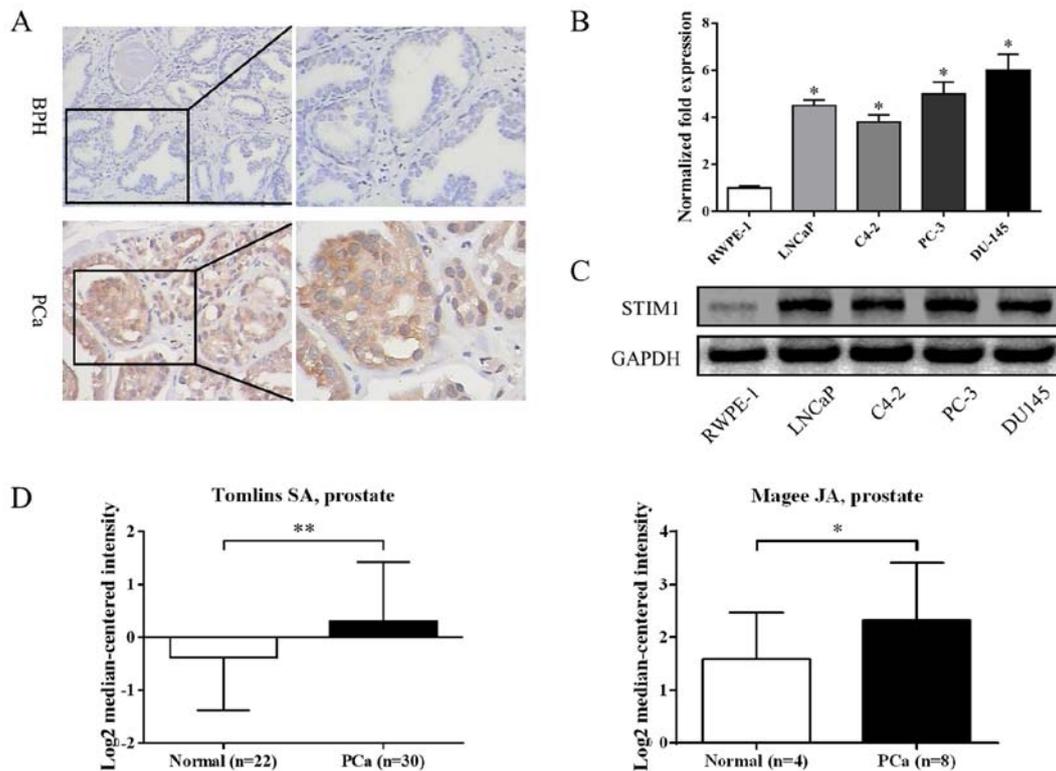


Figure 1. STIM1 is upregulated in PCa. (A) The expression of STIM1 in BPH tissues and prostate cancer tissues was detected by immunohistochemistry. High expression levels of STIM1 were observed in prostate cancer tissues. The expression of STIM1 was low in BPH tissues. (B and C) The expression of STIM1 in normal prostate cell line RWPE-1 and prostate cancer cell lines LNCaP, C4-2, PC-3 and DU-145 were determined by (B) RT-PCR at the mRNA level and (C) western blotting at the protein level. GAPDH was used as an internal control. (D) The expression of STIM1 in samples from the Oncomine database; \* $P < 0.05$ , \*\* $P < 0.01$ . STIM1, stromal-interacting molecule 1; PCA, prostate cancer; BPH, benign prostatic hyperplasia.

purchased from GeneChem Corporation (Shanghai, China). The targeting senses of the shRNAs were: shSTIM1-1, 5'-GCTCTCCACATTTGGATTCTT-3'; and shSTIM1-2, 5'-GGAGGATAATGGCTCTATT-3'. The negative control was a double-stranded shRNA without sequence homology to any known human genes. For gene silencing, purified lentiviruses (shSTIM1-1 and shSTIM1-2) were added to cells at a multiplicity of infection of 20 for 8 h, and was washed twice with medium. Infection with a multiplicity of infection of 20 resulted in a >90% infection of cancer cells after 72 h, as monitored by GFP expression. Therefore, we used a multiplicity of infection of 20 for the lentivirus in all of the experiments, as it yielded optimal knockdown of the gene in the required time. Control cells were infected with a negative control shRNA, as a vector control according to the same protocol.

**Wound healing assay.** Cells were seeded to 6-well plates at a concentration of  $5 \times 10^5$  cells/well and incubated overnight. The cells were then infected with lentiviruses and incubated for an additional 48 h until the cell monolayers formed. The cells were starved by serum-free medium overnight. The wounds were scratched by sterile 200  $\mu$ l pipet tips. After scratching, the cells were washed with PBS twice and cultured in serum-free medium. Images of the wounds were captured at time-points of 0, 12 and 24 h by an inverted microscope (magnification, x40).

**Transwell migration and invasion assays.** A Transwell migration assay was performed using Transwell chambers consisting

of 8  $\mu$ m membrane filter inserts (Corning, Corning NY, USA). Matrigel was diluted in serum-free medium (1:5) and pre-paved to insert membranes 4 h before cell seeding. The cells were infected with a lentivirus for 72 h, and then re-suspended in serum-free medium. The cells in 500  $\mu$ l serum-free medium ( $3 \times 10^4$  cells for migration and  $10 \times 10^4$  cells for invasion) were added to the upper chamber, and the lower chamber was filled with 1 ml normal culture medium containing 10% FBS. After incubation for 24 h, the cells were fixed with 4% paraformaldehyde and stained with crystal violet. Migrated and invaded cells were observed using a microscope (magnification, x100) and images were captured.

**Statistical analysis.** To carry out statistical analysis, we used the software SPSS 23 (SPSS, Inc., Chicago, IL, USA) and GraphPad Prism 6 (GraphPad Software, San Diego, CA, USA). The correlation between STIM1 expression and clinicopathological parameters was analyzed by Chi-square ( $\chi^2$ ) test. The rest of the experimental data were represented from at least 3 independent experiments. Statistical significance was evaluated using the Student's t-test. A P-value <0.05 was considered to indicate a statistically significant result.

## Results

**STIM1 is overexpressed in prostate cancer tissues.** To evaluate the expression of STIM1 in human prostate cancer tissues and benign prostate tissues (BPH), we examined the specimens by IHC. As shown in Fig. 1A, the expression of STIM1 in prostate

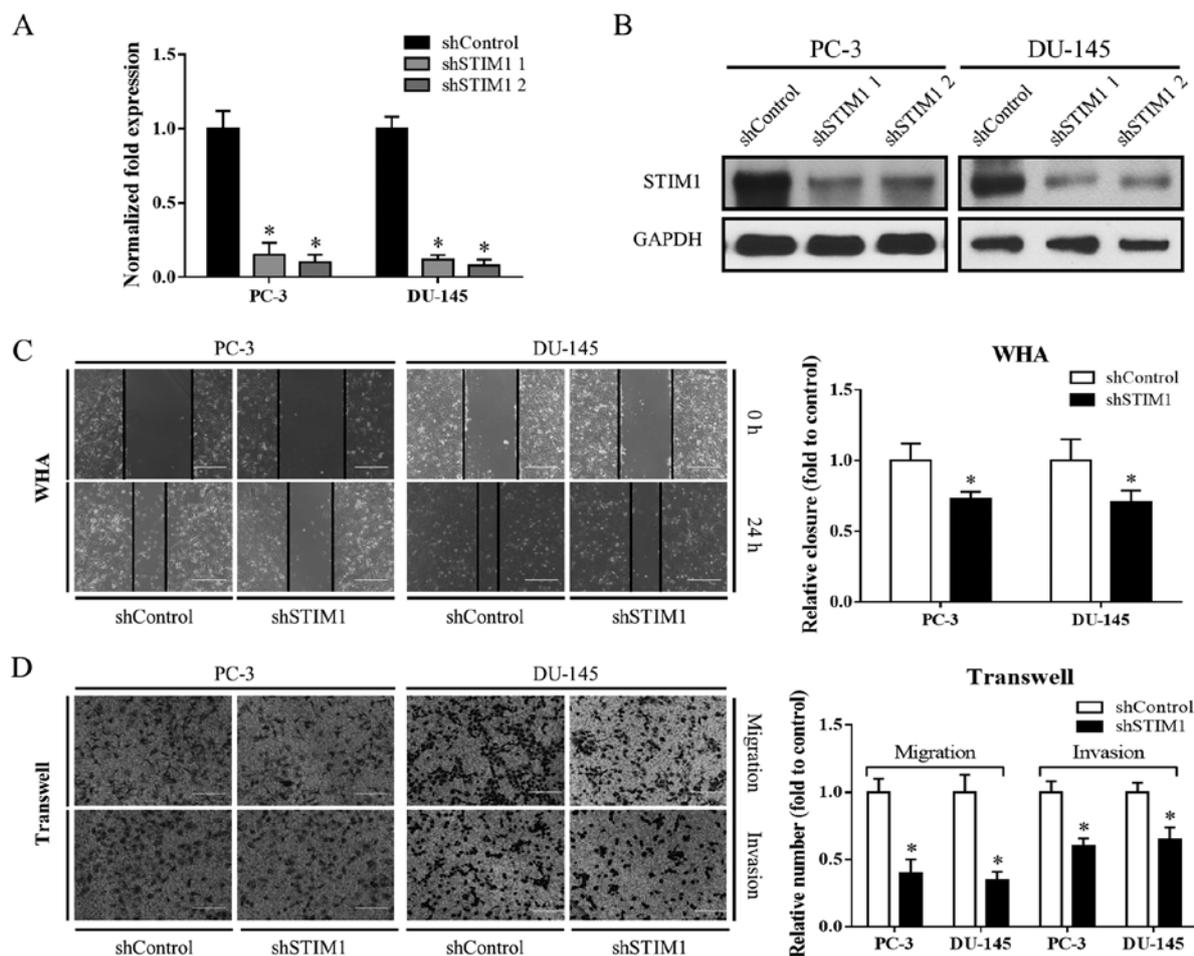


Figure 2. STIM1 knockdown inhibits cell migration and invasion. (A and B) Two independent STIM1 shRNAs and shControl were transfected into PC-3 and DU-145 cells. The knockdown efficiency was determined by (A) RT-PCR and (B) western blotting. (C and D) A wound healing assay and a Transwell assay were performed to study the influence of STIM1 knockdown on cell migration and invasion. The statistical analyses are shown in C and D;  $P < 0.05$ . STIM1, stromal-interacting molecule 1.

cancer was higher than benign prostatic hyperplasia (BPH) tissues. Overall, STIM1 was expressed in 3 out of 10 (30.0%) BPH specimens; whereas it was expressed in 37 out of 47 prostate cancer specimens (78.7%). The difference was statistically significant ( $P = 0.007$ ). We also searched in databases such as Oncomine ([www.oncomine.org](http://www.oncomine.org)) and found studies conducted by Magee *et al* (22) and Tomlin *et al* (23) which revealed similar results (Fig. 1D).

To study whether STIM1 was correlated to prostate cancer progression, the association between STIM1 expression and clinicopathological characteristics was analyzed. As shown in Table I, the expression of STIM1 was found to be significantly associated with stage grouping. The STIM1 expression level of stage I+II was significantly lower than that of stage III+IV ( $P = 0.016$ ). However, there was no significant correlation between STIM1 expression and age or prostate cancer Gleason score ( $P > 0.05$ ).

We also investigated the expression of STIM1 in cell lines derived from normal prostate or prostate cancers. As shown in Fig. 1B and C, the expression of STIM1 in prostate cancer cell lines was much higher than that in normal prostate epithelial cell line (RWPE-1) at both the mRNA and protein levels. These results indicate that STIM1 may be an oncogene in prostate tumorigenesis.

Table I. Association between STIM1 expression and clinicopathological features in patients recruited.

Characteristics	No.	STIM1 expression		P-values
		Weak	Strong	
Diagnosis				0.007
BPH	10	7	3	
PCa	47	10	37	
Age (PCa)				0.487
$\leq 65$	19	5	14	
$> 65$	28	5	23	
PCa Gleason score				0.709
$\leq 7$	14	2	12	
$> 7$	33	8	25	
PCa stage grouping				0.016
I+II	12	6	6	
III+IV	35	4	31	

STIM1, stromal-interacting molecule 1; BPH, benign prostatic hyperplasia; PCa, prostate cancer.

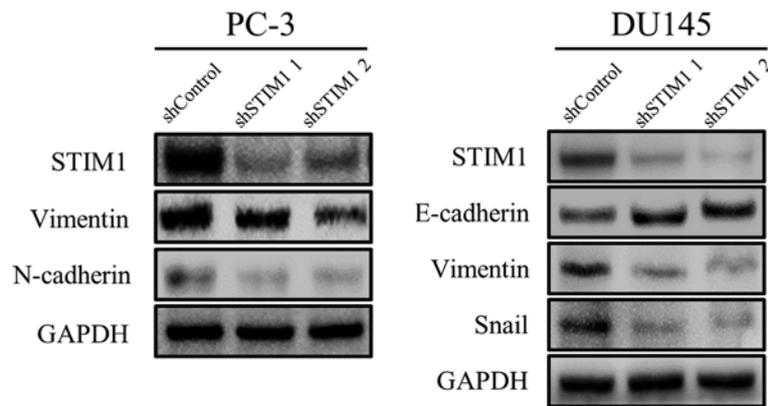


Figure 3. Motility inhibition by STIM1 knockdown is associated with EMT suppression. STIM1 was knocked down by two independent shRNAs in PC-3 and DU-145 cells. EMT markers E-cadherin, N-cadherin, vimentin and Snail were assessed by western blotting. STIM1, stromal-interacting molecule 1.

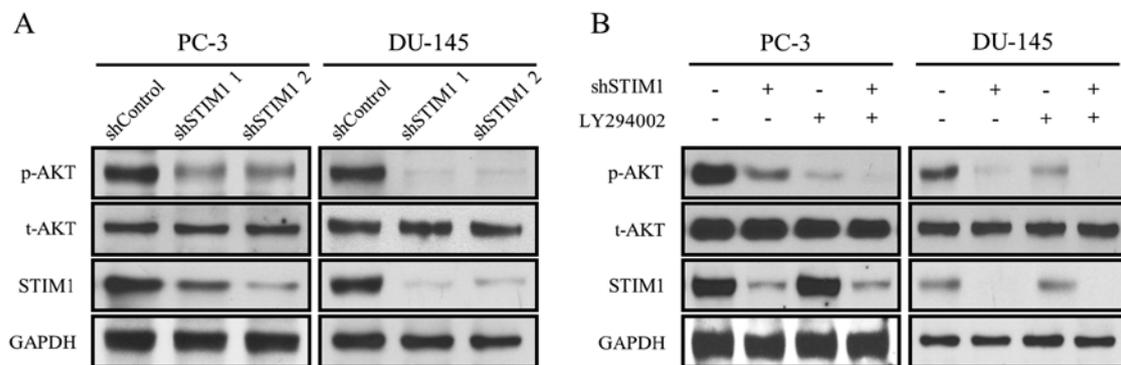


Figure 4. STIM1 knockdown inactivates PI3K-Akt. (A) Two independent shSTIM1s and shControl were transfected into PC-3 and DU-145 cells for 3 days and whole cell extracts were isolated. The expression of p-Akt, t-Akt and STIM1 was determined by western blotting. (B) shSTIM1 and shControl were transfected into PC-3 and DU-145 cells for 2 days, and then the culture medium was replaced with fresh medium with/without 10  $\mu$ M LY294002. Protein was harvested after 24 h of treatment and the expression of p-Akt, t-Akt and STIM1 was detected by western blotting. GAPDH was used as a loading control. STIM1, stromal-interacting molecule 1.

*STIM1 knockdown inhibits migration and invasion in prostate cancer cells.* To study the role of STIM1 in prostate cancer, we first designed two independent STIM1 shRNAs to knock down STIM1 in PC-3 and DU-145 cell lines. A double-stranded shRNA without sequence homology to any known human genes was used as control (Fig. 2A and B).

Wound healing assay and Transwell migration were then utilized to study the impact of STIM1 knockdown on cell migration. As shown in Fig. 2C and D, the STIM1 knockdown groups exhibited less wound closure and migrated cells as compared to the shControl, indicating that cell migration was inhibited when STIM1 was knocked down. Cell invasion was also suppressed by STIM1 knockdown as determined by Transwell invasion assay (Fig. 2D).

*Motility inhibition by STIM1 knockdown is associated with EMT suppression.* To understand the potential mechanism involved in the suppressed migration and invasion by STIM1 knockdown, we examined EMT-related markers since STIM1 has been reported to promote cancer cell metastasis through the induction of EMT. As shown in Fig. 3, vimentin and N-cadherin were downregulated by STIM1 knockdown in PC-3 cells. In DU-145 cells, STIM1 knockdown decreased the expression of vimentin and Snail but increased the expression

of E-cadherin. These results demonstrated that the migration and invasion inhibited by STIM1 knockdown were associated with EMT suppression.

*The PI3K/Akt signaling pathway is inactivated by STIM1 knockdown.* To study the influence of STIM1 knockdown on the PI3K/Akt signaling pathway, we assessed the expression of p-Akt, t-Akt and STIM1 in PC-3 and DU-145 cells after 72 h of infection with shSTIM1. As shown in Fig. 4A, p-Akt (Thr308) was suppressed by STIM1 knockdown with no change observed in t-Akt. To further confirm the involvement of PI3K/Akt, LY294002, a classic PI3K inhibitor, was used. As shown in Fig. 4B, both LY294002 and STIM1 decreased the levels of p-Akt without affecting the expression of t-Akt. When these two reagents were used in combination, the PI3K/Akt inhibition effect was further enhanced (Fig. 4B).

These results revealed that the PI3K/Akt signaling pathway was suppressed by STIM1 knockdown in prostate cancer cells.

*The PI3K/Akt signaling pathway is involved in the inhibition of migration and invasion induced by STIM1 knockdown.* To determine whether PI3K/Akt inactivation mediated the suppression of STIM1 knockdown on cell migration and invasion, we detected cell migration under the treatment of

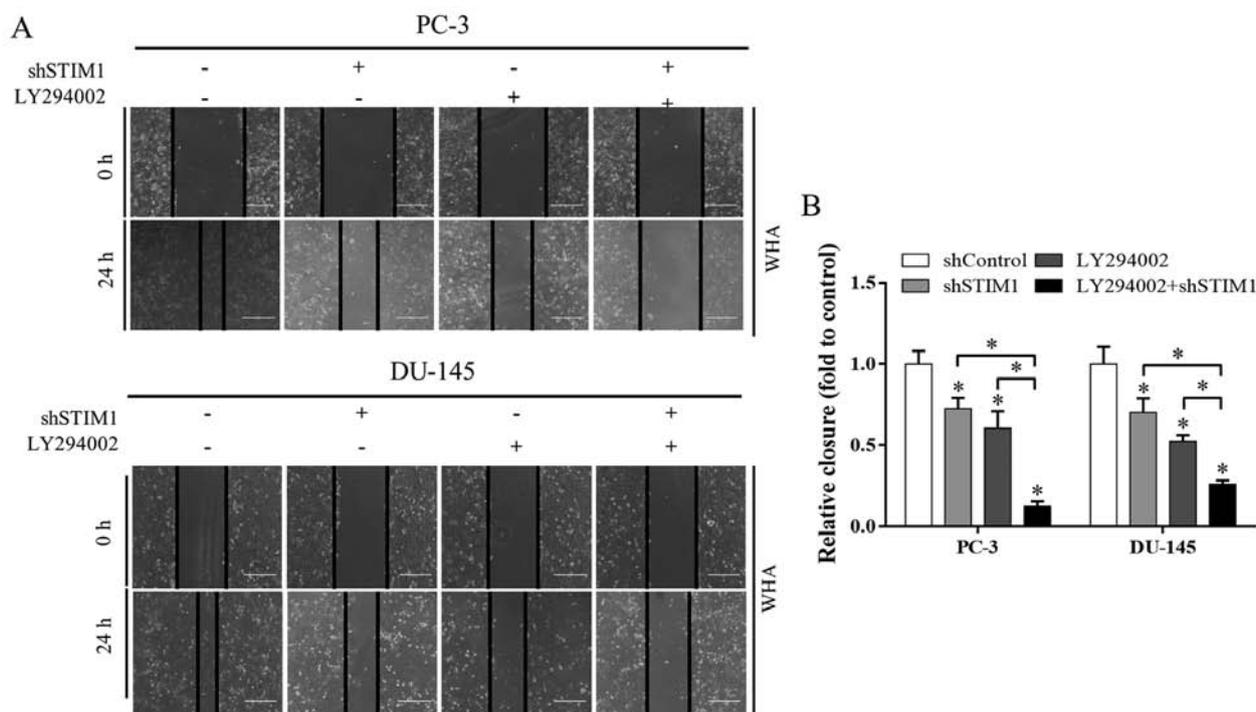


Figure 5. The PI3K/Akt signaling pathway is involved in the inhibition of migration and invasion induced by STIM1 knockdown. PC-3 and DU-145 cells were transfected with shSTIM1 and shControl for 48 h, and then treated with culture medium with/without 10  $\mu$ M LY294002 for an additional 24 h. (A and B) Cell migration was determined by wound healing assay; \* $P$ <0.05. STIM1, stromal-interacting molecule 1.

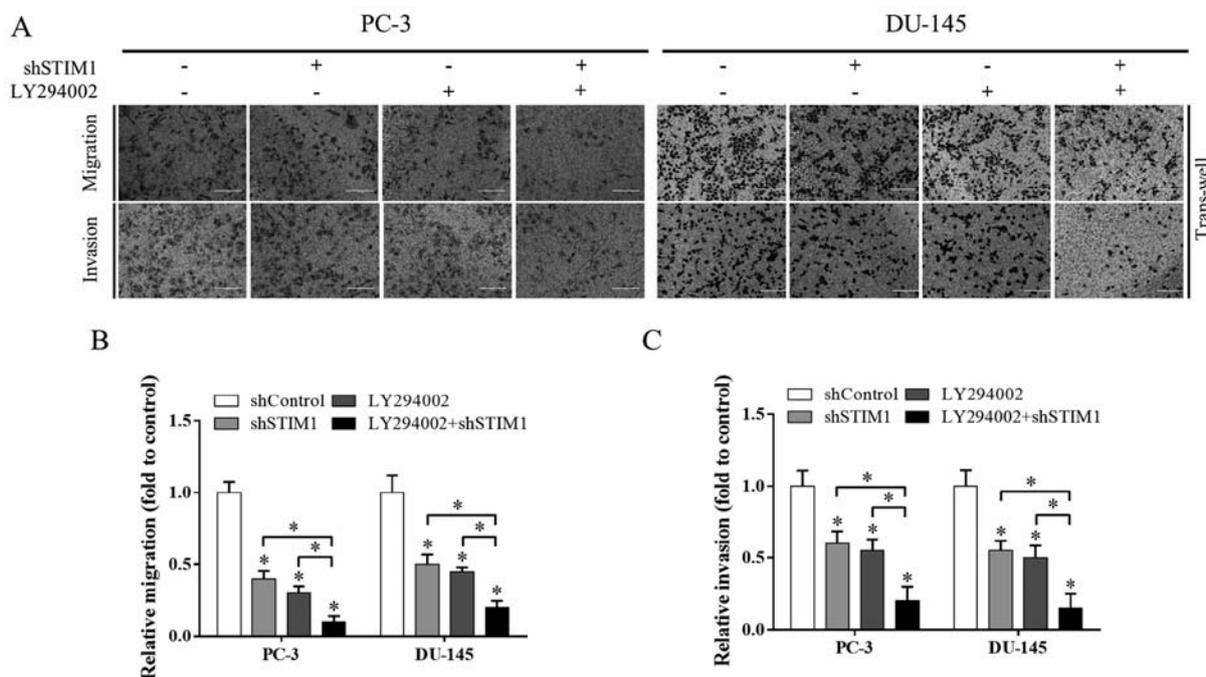


Figure 6. The PI3K/Akt signaling pathway is involved in the inhibition of migration and invasion induced by STIM1 knockdown. PC-3 and DU-145 cells were transfected with shSTIM1 and shControl for 48 h, and then treated with culture medium with/without 10  $\mu$ M LY294002 for an additional 24 h. (A and B) Cell migration was determined by Transwell migration assay. (A and C) Cell invasion was determined by Transwell invasion assay; \* $P$ <0.05. STIM1, stromal-interacting molecule 1.

LY294002 and/or STIM1 knockdown. Both LY294002 and STIM1 inhibited cell migration, and when these two treatments were used in combination, cell migration inhibition was markedly enhanced, as determined by wound healing assay (Fig. 5) and Transwell migration assay (Fig. 6A and B).

A similar phenomenon was observed in the Transwell invasion assay when studying cell invasion (Fig. 6A and C).

These data reveal that inactivation of PI3K/Akt is a potential underlying mechanism involved in migration and invasion inhibition induced by STIM1 knockdown.

## Discussion

Calcium signaling regulates a variety of cellular functions by activating or inhibiting cellular genes and signaling pathways (24,25). A number of studies have documented that tumor progression is generally associated with dysregulated expression of  $\text{Ca}^{2+}$  channels and other molecules involved in  $\text{Ca}^{2+}$  homeostasis (26-28). STIM1 is one of the important components of major  $\text{Ca}^{2+}$  entry in non-excitabile cells and has been reported to be aberrantly expressed in various types of cancer. STIM1 plays an important role in cell proliferation, migration and invasion in cervical cancer, hepatocarcinoma, glioblastoma and gastric cancer. Thus, STIM1 is considered to be an oncogene and a potential therapeutic target for these types of cancer (29,30). A recent study revealed that STIM1 was downregulated in prostate cancer (31), which was contrary to other studies and databases. Thus, there is a need to clarify the exact role of STIM1 in prostate cancer and further explore its functions. In the present study, we found that STIM1 was upregulated both in prostate cancer tissues and prostate cancer cell lines. STIM1 played a pivotal role in prostate cancer cell migration and invasion as STIM1 knockdown decreased both cell migration and invasion. We then examined EMT-related markers and found that mobility inhibited by STIM1 knockdown was associated with EMT reversion.

Increasing studies indicate that  $\text{Ca}^{2+}$  is an important regulator of the PI3K/Akt signaling pathway, and an increase of intracellular  $\text{Ca}^{2+}$  activates the PI3K/Akt pathway (32-37). PI3K/Akt signaling is one of the most important intracellular pathways regulating cellular homeostasis. PI3K/Akt is over-activated in a various types of cancer, and is associated with progression of malignancy and, in particular, metastasis. Inhibition of the PI3K/Akt pathway was revealed to suppress invasion and migration in a variety of cancer models and was considered to be a potential therapeutic approach to combat cancer metastases (38-40).

However, in prostate cancer, whether migration and invasion which were regulated by STIM1 involved the PI3K/Akt pathway is still uncertain. Our mechanistic study revealed that the PI3K/Akt signaling pathway was inactivated by STIM1 knockdown and was involved in the inhibition of migration and invasion induced by STIM1. In addition, the effect of STIM1 depletion was further enhanced by the combination with LY294002, suggesting that STIM1 knockdown inhibits the migration and invasion of prostate cancer cells involving the PI3K/Akt signaling pathway. However, besides the PI3K/Akt pathway, some other signaling pathways are also sensitive to calcium level changes within cells, and have been reported to be regulated by STIM1, such as the MAPK and AMPK pathways. Knockdown of STIM1 attenuates cytosolic calcium and subsequently increases AMPK phosphorylation (41). Furthermore, AMPK is a regulator of STIM1. STIM1 phosphorylation was reported to be mediated by the AMPK-p38 MAPK signaling axis (42). These signaling pathways differ from each other, but also have cross-talks. They work together to form a complex network while STIM1 is an important trigger. Our study just elucidated a part of the picture and more light is needed to be shed on the detailed mechanism involved in the future.

Collectively, our results indicated that STIM1 knockdown inhibited the migration and invasion of prostate cancer cells

involving the inactivation of PI3K/Akt signaling pathway. These findings shed new light on our understanding of STIM1 and suggest that STIM1 may be a potential target in the prevention of prostate cancer metastasis.

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