

# Thapsigargin induces apoptosis of prostate cancer through cofilin-1 and paxillin

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**Abstract.** It is widely considered that endoplasmic reticulum stress may rapidly induce apoptosis. The aim of the present study was to investigate the effect of thapsigargin on the induction of apoptosis in prostate cancer cells, and to explore its possible mechanism. A Cell Counting Kit-8 was selected to determine the effect of thapsigargin (0, 1, 10 and 100 nM) on the proliferation of PC3 cells. Cell proliferation of the prostate cancer cells was effectively inhibited by treatment with thapsigargin, and thapsigargin significantly increased the rate of apoptosis and caspase-3/9 activities in prostate cancer cells. The protein expression of phosphorylated (p)-RAC- $\alpha$  serine threonine-protein kinase, p-mechanistic target of rapamycin, F-actin and paxillin were significantly decreased, and cofilin-1 protein expression was significantly increased by treatment with thapsigargin in prostate cancer cells. Overall, the data of the present study revealed that thapsigargin induced apoptosis in prostate cancer cells through cofilin-1 and paxillin.

## Introduction

Prostate cancer is one of the most common types of cancer in men and the mortality rate was 9.37/100,000 in 2012, which has increased yearly in China (1). An increasing amount of evidence has indicated that age, ethnicity and family history of prostate cancer are factors associated with morbidity of prostate cancer (2). The occurrence and development of prostate cancer may be the result of the interaction between genetic predisposition and environmental factors, while genetic variation determines the susceptibility of individuals to suffer from prostate cancer (3). At present, the involvement of whole-genome analysis of different

ethnic groups worldwide has revealed that there are >30 susceptible sites on the genome associated with the risks of prostate cancer occurrence (4). The study of a genetic predisposition to prostate cancer, has demonstrated notable results (4,5).

The apoptosis signaling pathway is stimulated by exogenous and endogenous stresses (6). These specific stresses are: Unfolded protein accumulation in the endoplasmic reticulum; disordered ingestion and release of endoplasmic reticulum calcium; and incorrect processing of specific proteins, either accompanied or unaccompanied, in the endoplasmic reticulum (6). Sustained endoplasmic reticulum stress results in apoptosis (7). Caspase-3 is a member of the caspase protein family, and is located in the endoplasmic reticulum (8). The specificity of caspase-3 may be activated by endoplasmic reticulum stress.

Cofilin is a type of actin binding protein in eukaryons with a low molecular weight (9). The *cofilin-1* gene is positioned at chromosome 11q13 and is expressed in non-muscular tissues (9). F-actin regulates the reconstruction of the actin framework and moves cells forward by reconstructing the schistose pseudopodia and lamellar structures of the front histiocyte (10). Highly activated cofilin-1 has been demonstrated in glioma, Lymphocytoma cutis, colon cancer, hepatoma carcinoma, renal carcinoma, esophageal squamous cancer and prostate cancer cells (11).

Paxillin is a phosphoric acid protein, with a molecular mass of 68x10<sup>3</sup>, and its main role is in the process of focal adhesion (12). It functions to combine vinculin and actin (12). The human paxillin gene is positioned at chromosome 12q24, and there are 11 expressed regions (13). The paxillin molecule contains multiple structural domains and a combination of a series of signal proteins and structural proteins to mediate cell signaling transduction (13). It serves an important role in cell adhesion and transport processes, and has a close association with localized cancer cell movement (13).

It is widely hypothesized that thapsigargin (Fig. 1) induces endoplasmic reticulum stress, and may induce multiple cells to undergo endoplasmic reticulum stress and apoptosis (14). It has been demonstrated that thapsigargin may inhibit A549 cell growth, the source of which type II alveolar epithelial cells, and induce apoptosis (14). Concurrently, thapsigargin

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may induce leukemic K562 cells to undergo apoptosis in response to endoplasmic reticulum stress (15). The action of thapsigargin is to suppress activity of the endoplasmic reticulum membrane  $\text{Ca}^{2+}$ -adenosine 5'-triphosphate enzyme, induce the increase in concentration of intracytoplasmic  $\text{Ca}^{2+}$  and reduce the level of stored calcium in the endoplasmic reticulum, so as to cause endoplasmic reticulum stress and induce apoptosis (16). The present study explored whether thapsigargin induced apoptosis in prostate cancer cells, and explored its possible mechanism.

## Materials and methods

**Cell culture.** The human prostate cancer PC3 cell line was purchased from the Shanghai Cell Bank of The Chinese Academy of Sciences (Shanghai, China) and maintained in RPMI-1640 media (Hyclone; GE Healthcare Life Sciences, Logan, UT, USA) containing 10% fetal bovine serum (Hyclone; GE Healthcare Life Sciences) and 100  $\mu\text{g}/\text{ml}$  penicillin-streptomycin (Invitrogen; Thermo Fisher Scientific, Inc., Waltham, MA, USA) at 37°C and 5%  $\text{CO}_2$ .

**Cell proliferation assay.** The Cell Counting Kit-8 (CCK-8, Beyotime Institute of Biotechnology, Haimen, China) was selected to determine the effect of thapsigargin (0, 1, 10 and 100 nM) on PC3 cell proliferation. The PC3 cells were seeded in 96-well plates at a density of  $1 \times 10^4$  cells/well and incubated with thapsigargin (0, 1, 10 and 100 nM) for 12, 24 and 48 h at 37°C. Then, 10  $\mu\text{l}$  CCK-8 reagent was added and incubated for 4 h at 37°C. Cell proliferation was detected at 450 nm using a microplate reader (Bio-Rad Laboratories, Inc., Hercules, CA, USA).

**Flow cytometric analysis for cell apoptosis.** The PC3 cells were seeded in 6-well plates at a density of  $1 \times 10^6$  cells/well and incubated with thapsigargin (0, 1, 10 and 100 nM) for 24 h at 37°C. The PC3 cells were washed with cold PBS twice, and re-suspended with 500  $\mu\text{l}$  binding buffer (BD Biosciences, Franklin Lakes, NJ, USA). Then, 5  $\mu\text{l}$  Annexin V-fluorescein isothiocyanate (BD Biosciences) was added to the cells and incubated for 30 min at 4°C in the dark. FACSCalibur flow cytometry (BD Biosciences) was performed following the addition of 10  $\mu\text{l}$  propidium iodide for 15 min in the dark at room temperature and FlowJo software (version 7.6.1; (FlowJo LLC, Ashland, OR, USA) was used to analyze apoptosis rate.

**Caspase-3 and caspase-9 activity analysis.** The PC3 cells were seeded in 96-well plates at  $1 \times 10^4$  cells/well and incubated with thapsigargin (0, 1, 10 and 100 nM) for 24 h at 37°C. A total of 100  $\mu\text{l}$  caspase-3 or caspase-9 reagent (C1136 or C1158, Beyotime Institute of Biotechnology) was added and incubated at room temperature for an additional 2 h. Caspase-3 and caspase-9 activity was detected at 490 nm using a microplate reader (Bio-Rad Laboratories, Inc.).

**Western blot analysis.** The PC3 cells were seeded in 6-well plates at  $1 \times 10^6$  cells/well and incubated with thapsigargin (0, 1, 10 and 100 nM) for 24 h at 37°C. Then, the PC3 cells were washed with cold PBS twice and prepared using a ProteoJET

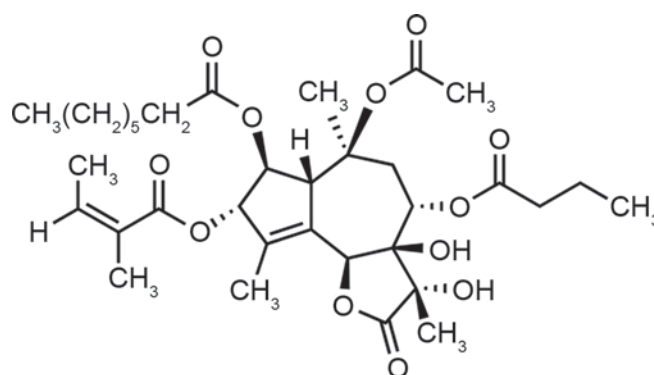


Figure 1. Chemical structure of thapsigargin.

cytoplasmic protein extraction kit (Fermentas; Thermo Fisher Scientific, Inc.). Protein concentrations were measured using a BCA Protein Assay kit (Thermo Fisher Scientific Inc.). Protein (30  $\mu\text{g}$ ) was separated by 10-12% SDS-PAGE and transferred electrophoretically using a PVDF membrane by standard procedures. The PVDF membrane was blocked for 2 h with 5% non-fat milk in TBST (TBS + 0.1% Tween-20) at 37°C and probed overnight using the following primary antibodies: Anti-RAC- $\alpha$  serine threonine-protein kinase (Akt, sc-135829; 1:1,000; Santa Cruz Biotechnology, Inc., Dallas, TX, USA); anti-phosphorylated (p)-Akt (sc-7985-R; 1:500; Santa Cruz Biotechnology, Inc.); anti-p-mechanistic target of rapamycin (p-mTOR; sc-101738; 1:500; Santa Cruz Biotechnology, Inc.); anti-F-actin (ab205; 1:1,000; Santa Cruz Biotechnology, Inc.); anti-cofilin-1 (sc-376476; 1:1,000; Santa Cruz Biotechnology, Inc.); anti-paxillin (sc-390738; 1:1,000; Santa Cruz Biotechnology, Inc.); and anti- $\beta$ -actin (sc-1616; 1:2,000; Santa Cruz Biotechnology, Inc.), in PBST at 4°C. Then, the membrane was incubated with horseradish peroxidase-conjugated goat anti-rabbit secondary antibody (1:2,000 dilution; sc-2004 or sc-2005, Santa Cruz Biotechnology, Inc.) for 2 h at room temperature and detected by Super Signal enhanced chemiluminescence development (ECL) reagent (Pierce; Thermo Fisher Scientific, Inc.) and analyzed using sodium Image Lab software (version 3.0; Bio-Rad Laboratories, Inc.).

**Statistical analysis.** Data are expressed as mean  $\pm$  standard deviation and were analyzed using the SPSS version 17.0 software (SPSS, Inc., Chicago, IL, USA). A one-way analysis of variance and Tukey's post-hoc test was used to compare data between groups.  $P < 0.05$  was considered to indicate a statistically significant difference.

## Results

**Thapsigargin inhibits cell proliferation in prostate cancer cells.** The present study demonstrated that thapsigargin may suppress cell proliferation of prostate cancer PC3 cells in a dose- and time-dependent manner (Fig. 2). Treatment with 10 and 100 nM thapsigargin at 24 or 48 h or 1, 10 and 100 nM thapsigargin at 12 h significantly suppressed cell proliferation of PC3 cells, compared with 0 nM of thapsigargin (Fig. 2).

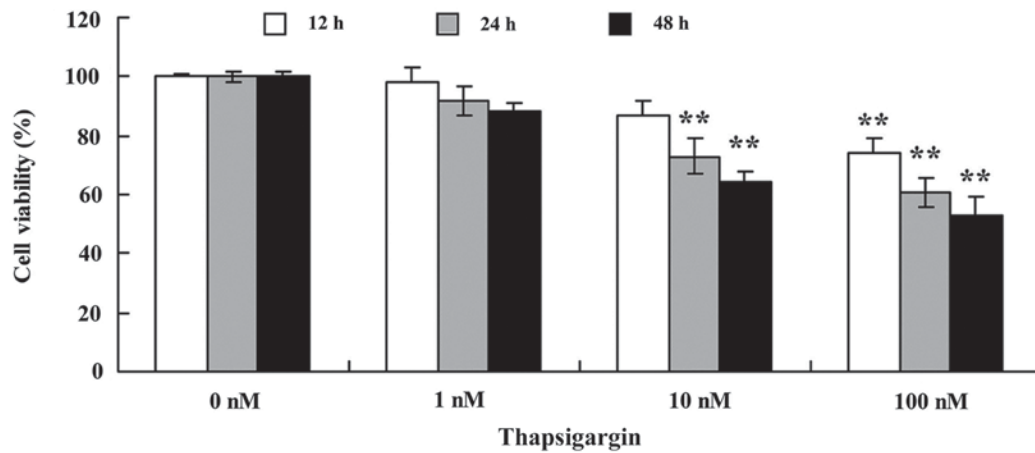


Figure 2. Thapsigargin inhibits cell proliferation in prostate cancer cells. \*\*P<0.01 compared with 0 nM thapsigargin group.

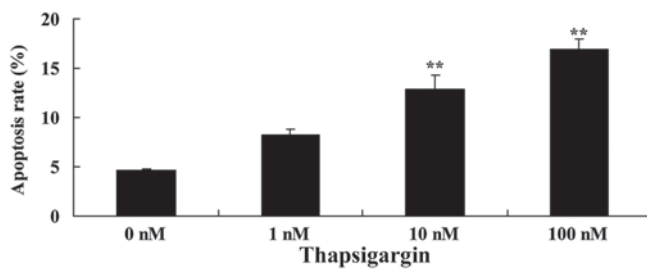


Figure 3. Thapsigargin induces apoptosis rate of prostate cancer cells. \*\*P<0.01 compared with 0 nM thapsigargin group.

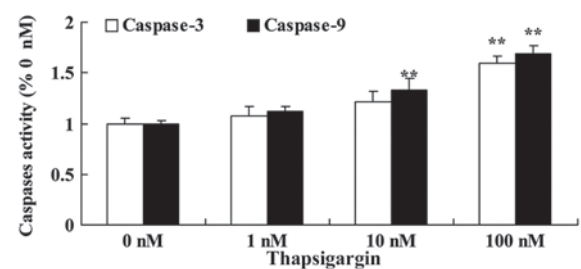


Figure 4. Thapsigargin induces caspase-3/and 9 activities of prostate cancer cells. \*\*P<0.01 compared with 0 nM thapsigargin group.

*Thapsigargin increases the apoptosis rate in prostate cancer cells.* Consistent with the aforementioned data, 10 and 100 nM thapsigargin significantly increased the apoptosis rate of prostate cancer PC3 cells in a dose-dependent manner (Fig. 3). These data suggest that thapsigargin may suppress cell proliferation and increase the apoptosis rate of PC3 cells as a potential treatment for prostate cancer.

*Thapsigargin induces caspase-3/9 activities in prostate cancer cells.* To explore the anticancer effects of thapsigargin on cell apoptosis, caspase-3/9 activities in PC3 cells were then examined. Treatment with 10 and 100 nM thapsigargin significantly increased caspase-9 activities, and treatment with 100 nM thapsigargin significantly increased caspase-3 activities in PC3 cells compared with the 0  $\mu$ M thapsigargin group (Fig. 4).

*Thapsigargin inhibits Akt in prostate cancer cells.* To confirm the potential mechanism of thapsigargin action, p-Akt and Akt protein expression levels were investigated by western blot analysis. As demonstrated in Fig. 5A and B, p-Akt protein expression was significantly decreased in PC3 cells by treatment with 10 and 100 nM thapsigargin compared with the 0 nM treatment group.

*Thapsigargin inhibits mTOR expression in prostate cancer cells.* Next, the p-mTOR expression level in prostate cancer cells was examined by treatment with thapsigargin in PC3 cells. Treatment with 10 and 100 nM thapsigargin significantly

decreased p-mTOR protein expression in PC3 cells compared with the 0  $\mu$ M thapsigargin group (Fig. 5C).

*Thapsigargin inhibits F-actin expression in prostate cancer cells.* To determine the functional significance of F-actin in the regulation of the effect of thapsigargin on prostate cancer cells, F-actin protein expression was analyzed using western blot analysis. The western blot analysis data from the present study indicated that F-actin protein expression was significantly decreased by treatment with 100 nM thapsigargin in PC3 cells compared with the 0  $\mu$ M thapsigargin group (Fig. 6A and B).

*Thapsigargin induces cofilin-1 expression in prostate cancer cells.* Furthermore, the effect of thapsigargin on cofilin-1 expression in prostate cancer cells was determined using western blot analysis. As indicated in Fig. 6A and C, treatment with 100 nM thapsigargin significantly increased cofilin-1 protein expression in prostate cancer PC3 cells compared with the 0  $\mu$ M thapsigargin group (Fig. 6A and C).

*Thapsigargin inhibits paxillin expression in prostate cancer cells.* To additionally confirm the inhibitory effect of thapsigargin on paxillin expression in prostate cancer cells, the protein expression of paxillin was measured using western blot analysis. Treatment with 10 and 100 nM thapsigargin significantly decreased paxillin protein expression in prostate cancer PC3 cells compared with the 0  $\mu$ M thapsigargin group (Fig. 6D).

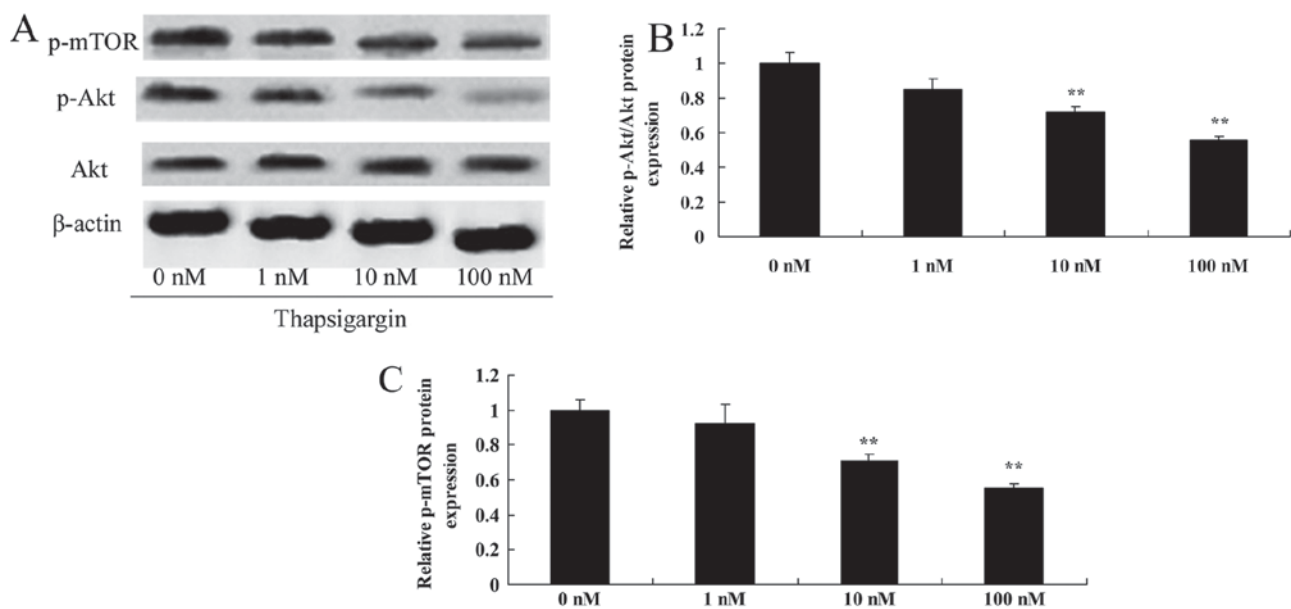


Figure 5. Thapsigargin inhibits PI3K/Akt of prostate cancer cells. Thapsigargin inhibits p-Akt/Akt protein expression by (A) western blot assays and (B) densitometric analysis of p-Akt/Akt protein expression of prostate cancer cells. (C) Thapsigargin inhibits p-mTOR protein expression. \*\* $P < 0.01$  compared with 0 nM thapsigargin group.

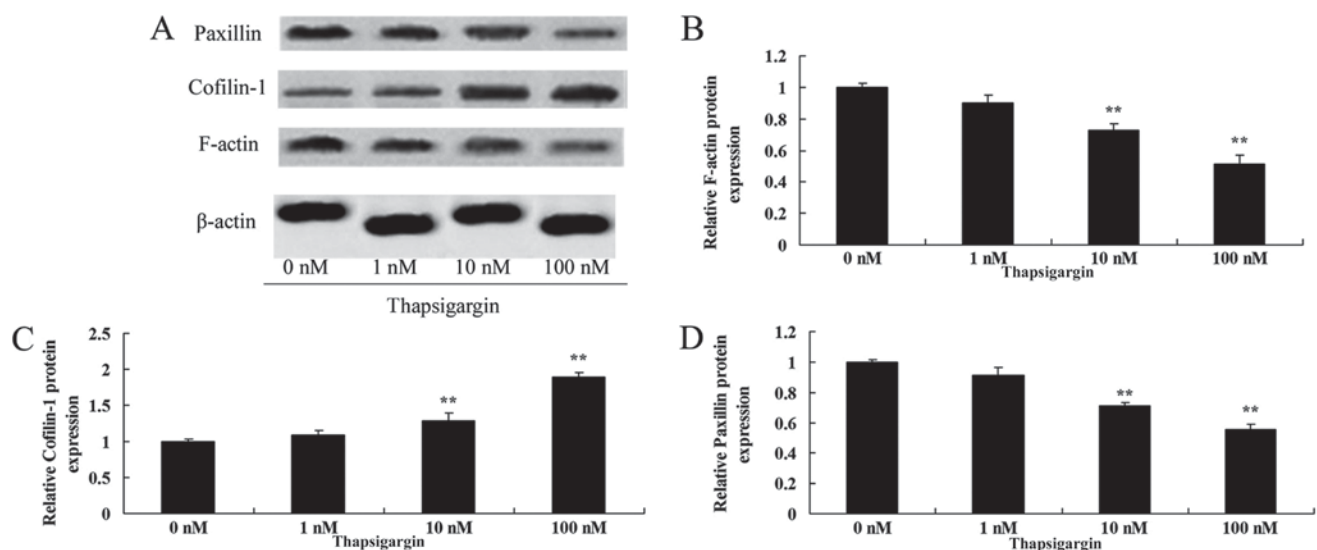


Figure 6. Thapsigargin inhibits F-actin, cofilin-1 and paxillin expression in prostate cancer cells. (A) Western blot analysis of the protein expression levels of F-actin, cofilin-1 and paxillin. (B) Densitometric analysis of F-actin expression. (C) Densitometric analysis of cofilin-1 expression. (D) Densitometric analysis of paxillin expression. \*\* $P < 0.01$  compared with 0 nM thapsigargin group.

## Discussion

Prostate cancer is one of the most common types of malignant tumors. Its mortality rate ranks sixth globally (17). There are ~903,500 incident cases globally every year, including 258,400 mortalities (17). Despite the improvement of diagnostic technology and effective development of screening processes, the morbidity of prostate cancer in Asian and European countries including China in recent years has increased (18). However, during the progression of treatment, it is inevitable for patients with prostate cancer to develop resistance to hormone therapy within several years, namely castrate-resistant prostate cancer (17). For

hormone-independent prostate cancer, which is insensitive to endocrinotherapy, there is no consistent ideal therapeutic method; therefore, it has become an increasingly difficult issue (4). The activation of caspases associated with endoplasmic reticulum stress occurs during the early phase of apoptosis, when cells suffer from a stress reaction, resulting in increases in mitochondrial outer membrane permeability (19). Cytochrome *c* is released into the cytoplasm, which then promotes the formation of the apoptosis complex, activates effector caspases and causes apoptosis (8). The present study indicated that thapsigargin significantly decreased cell proliferation, and increased the apoptosis rate and caspase-9/3 activities in PC3 cells.



The mTOR signal transduction pathway primarily participates in the synthesis of proteins (20). A previous study concerning the suspected associations between single nucleotide polymorphisms in the signal transduction pathway gene and prostate cancer conducted worldwide (21). The mTOR signaling pathway is an important therapeutic target of prostate cancer (21). mTOR is a highly conserved serine/threonine kinase, belonging to the phosphoinositide 3-kinase (PI3K) family, and is also the downstream effector of the PI3K/Akt signaling pathway (21). mTOR is widely expressed in cells and regulates multiple cellular functions in different cells, including survival and proliferation (21). On the one hand, the mammalian target of rapamycin complex 1 regulates translation, including 5'terminal oligopyrimidine tract mRNAs (20). Conversely, mTORC1 serves as the central pivot of the cascade signal channel that regulates RNA translation. The present study identified that thapsigargin inhibits p-Akt and p-mTOR protein expression in prostate cancer cells. Chiu *et al* (22) demonstrated that thapsigargin induces pro-death autophagy through Akt-mTOR-Ribosomal protein S6 kinase  $\beta$ -1 pathway inhibition in multidrug-resistant lung cancer cells. These data suggest the role of thapsigargin-mediated inhibition of the Akt-mTOR pathway in prostate cancer cells.

The cytoskeleton is a network structure consisting of cellular internal proteins, including canaliculi, microfilaments and intermediate filaments (23). Microfilaments are the smallest of the three skeleton structures, are composed of actin and exist in the form of free and globular actin G-actin or F-actin (23). Previous data indicate that the changes in actin polymerization/depolymerization, namely actin skeleton reconstruction, serve important regulatory roles in phenotypes of malignant cells (24). It has been suggested that intervention in cellular skeleton microfilament actin reconstruction may be a functional target of anticancer drugs, and may be regarded as a basis for developing novel antineoplastic drugs (25). Yip *et al* (26) suggested that thapsigargin modulates osteoclastogenesis through the regulation of F-actin and reactive oxygen species production (27). The present study indicated that thapsigargin inhibits F-actin expression in prostate cancer cells.

The highly localized activities of cofilin-1 generate schistose pseudopodia and determine cellular motor direction; cofilin-1 serves an important role in cell migration (9). Concomitantly, there have been studies demonstrating that cofilin-1 is an important regulatory factor of cancer cell metastasis and invasion (9). The overexpression of cofilin-1 protein levels increases the migratory rate of cancer cells, but inhibiting its expression may markedly reduce cancer cell growth (9). An overexpression of cofilin-1 at a mRNA level has been revealed in breast cancer cell subsets (11). A previous study identified that cofilin-1 also exhibited overexpression in a number of types of cancer cells (11). The present study suggested that thapsigargin induces cofilin-1 expression in prostate cancer cells. Wang *et al* (27) demonstrated that thapsigargin induces apoptosis of human lung adenocarcinoma cells through cofilin-1 and paxillin.

Protein-tyrosine kinase 6 (Brk) promotes the migration and infiltration of prostate cancer, and is activated by paxillin phosphorylation (10). Paxillin is the binding protein and acting substrate of Brk (10). Then, the adaptor molecule CrkII isoform activates guanosine triphosphatase Ras-related C3 botulinum

toxin substrate 1, to cause cell migration and infiltration (10). In addition, paxillin may also combine with multiple oncogenic proteins, disordering or even dysregulating normal adhesion, and control the growth factor signaling passageway required for cell proliferation, so as to participate in tumor metastasis (23). The abnormal expression of paxillin is associated with the occurrence, invasion and metastasis of prostate cancer (23). The present study identified that thapsigargin significantly inhibited paxillin protein expression in prostate cancer PC3 cells. Wang *et al* (27) revealed that thapsigargin induces apoptosis in human lung adenocarcinoma cells through cofilin-1 and paxillin. These results of the present study suggest that curcumin inhibits the tumor growth of prostate cancer cells by modulating the F-actin/cofilin-1/paxillin pathway.

In summary, the present study suggests that thapsigargin significantly decreased cell proliferation, and increased the apoptosis rate and caspase-9/3 activities in PC3 cells. Additionally, inhibition of Akt-mTOR pathway and modulation of the F-actin/cofilin-1/paxillin pathway by thapsigargin may decreased cell growth in prostate cancer cells. These data suggest that thapsigargin may be a novel drug that suppresses the growth of prostate cancer cells through the Akt-mTOR and F-actin/cofilin-1/paxillin pathways.

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#### Availability of data and materials

The analyzed data sets generated during the study are available from the corresponding author on reasonable request.

#### Authors' contributions

XW designed the experiment. FH and PW performed the experiments. XW and FH analyzed the data. XW wrote the manuscript.

#### Ethics approval and consent to participate

Not applicable.

#### Consent for publication

Not applicable.

#### Competing interests

The authors declare that they have no competing interests.

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