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TRPM7 is required for ovarian cancer cell growth, migration and invasion

Jing Wang [a], Qian-jin Liao [a], Yi Zhang [b], Hui Zhou [a], Chen-hui Luo [a], Jie Tang [a], Ying Wang [a], Yan Tang [a], Min Zhao [a], Xue-heng Zhao [a], Qiong-yu Zhang [c], Ling Xiao [d,e,*]

[a] The Affiliated Cancer Hospital of Xiangya School of Medicine, Central South University, Changsha 410013, PR China
[b] Department of Obstetrics and Gynaecology, Xiangya Hospital, Central South University, Changsha 410078, PR China
[c] Department of Basic Medical Science, Yongzhou Vocational Technical College, Yong Zhou 425100, PR China
[d] Department of Histology and Embryology, School of Basic Medical Sciences, Central South University, Changsha 410013, PR China
[e] Institute of Clinical Pharmacology, Central South University, Changsha 410018, PR China

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ABSTRACT

Our previous study demonstrated that the melastatin-related transient receptor potential channel 7 (TRPM7) was highly expressed in ovarian carcinomas and its overexpression was significantly associated with poor prognosis in ovarian cancer patients. However, the function of TRPM7 in ovarian cancer is mostly unknown. In this study, we examined the roles of TRPM7 in ovarian cancer cell proliferation, migration and invasion. We found that short hairpin RNA interference-mediated silence of TRPM7 significantly inhibited cell proliferation, colony formation, migration and invasion in multiple ovarian cancer cell lines. Mechanistic investigation revealed that silence of TRPM7 decreased phosphorylation levels of Akt, Src and p38 and increased filamentous actin and focal adhesion number in ovarian cancer cells. Thus, our results suggest that TRPM7 is required for proliferation, migration and invasion of ovarian cancer cells through regulating multiple signaling transduction pathways and the formation of focal adhesions.

1. Introduction

As one of the most common cancer in women, ovarian cancer is the most lethal gynaecological cancer among females in the world [1]. Although our basic knowledge regarding ovarian cancer has been greatly improved and more aggressive surgery and chemotherapy have been applied, the mortality statistics has been only modest changed over the last decade. The main reason for the high mortality of ovarian cancer is that early symptoms of this cancer are silent and most of the patients have advanced stage of the disease at diagnosis [2]. In advanced stage, ovarian tumor cells can easily either directly metastasize into neighboring organs such as bladder and colon or be transported throughout the peritoneum by peritoneal fluid within the abdominal cavity [3]. In addition, the development of multidrug resistances in ovarian cancer is another reason for the failure of the cancer treatment [4,5]. Thus, identifying more accurate molecular markers for prognosis and novel targets for the treatment are important for improving therapeutic methods and extending survival of ovarian cancer patients.

The transient receptor potential (TRP) channels are transmembrane proteins that play key roles in various physiologic and pathologic processes through modulating intracellular ion homeostasis [6]. As a member of TRP channel family, the melastatin transient receptor potential channel 7 (TRPM7) is both function as a Mg^{2+}/Ca^{2+}-permeable channel and a protein kinase that widely expresses in various normal tissues [7,8]. TRPM7 can phosphorylate itself and regulates many cellular processes including cell proliferation, adhesion, migration and survival. Recently, TRPM7 has been reported to aberrantly express in various types of cancers and be involved in carcinogenesis [9]. It has been found that overexpression of TRPM7 was associated with breast cancer progression and metastasis, whereas silence of TRPM7 inhibited cell proliferation, migration and invasion in various types of tumor cells [10–13]. Moreover, it have been shown to inhibit cell growth and induce apoptosis in breast cancer and gastric cancer cells, which further suggested that TRPM7 may serve as a potential therapeutic target for these cancers [14,15]. In our previous study, we have found that TRPM7 highly expressed in ovarian cancer and higher expression of TRPM7 was significantly associated with pelvic metastasis and

Abbreviations: TRPM7, transient receptor potential cation channel, subfamily M, member 7; OVCA, ovarian cancer.

* Corresponding author at: Department of Histology and Embryology, School of Basic Medical Sciences, Central South University, Changsha 410013, PR China.
E-mail address: lingxiaocsu@126.com (L. Xiao).

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poor prognosis in patients with ovarian cancer [16]. However, the function of TRPM7 in ovarian cancer is mostly unknown. In this study, our goals are to characterize the roles and possible mechanisms of TRPM7 mediating cell proliferation, migration and invasion in ovarian cancer cells.

2. Materials and methods

2.1. Reagents

Antibodies to TRPM7 (S74-25) was purchased from Cayman Chemical Company (Ann Arbor, USA). Antibody to Akt, p38, Src, phospho-Akt(S473), phospho-p38 and phospho-Src were purchased from Cell Signaling Technology (Beverly, MA). Antibody to vinculin (SPM227) was purchased from Abcam (Cambridge, USA). Alexa Fluor® 568 goat anti-mouse antibody and Alexa Fluor® 488 Phalloidin were purchased from Invitrogen (Grand Island, USA).

2.2. Cell lines

The human ovarian cancer cell lines SKOV-3, SW626 and PA-3 were purchased from ATCC and cultured at 37 °C with 5% CO_2 in DMEM Medium supplemented with 10% fetal bovine serum, 100 units/ml penicillin G, 100 μg/ml streptomycin, and 2 mM L-glutamine (Invitrogen).

2.3. Generating TRPM7 knocked down cells

The lentiviral silencing vector expressing shRNA targeting the human TRPM7 was obtained from Sigma (Sigma, MO). The colon IDs for TRPM7#1-shRNA and TRPM7#2-shRNA are TRCN0000021559 and TRCN0000021560, respectively. The empty lentiviral pLKO.1 vector was used as control. To generate lentivirus, HEK293T cells were cotransfected with pCMV-VSV-G and pCMV-dr8.2-dvpr and either pLKO.1 vector or TRPM7-shRNA plasmids. After 24 h, supernatant was collected and this lentiviral preparation was used to infect cells. After 24 h of infection, cells were selected with puromycin for an additional 48 h.

2.4. Cell growth curve

5×10^4 cells were seeded into 6-well plate and cultured for 6 days. Every 2 days, cells were harvested by trypsin and centrifuged for 5 min (1200 rpm). The supernatant was discarded and the cell pellet was re-suspended in an appropriate volume of media for cell counting by Trypan Blue. Three independent experiments were performed and the average number of the cells was plotted.

2.5. Western blotting

Cells were lysed in M2 lysis buffer (150 mM NaCl, 50 mM Tris-Cl (pH 8.0), 5 mM EDTA, 1% Nonidet P-40) containing a protease inhibitor mixture (Roche Applied Science) and a phosphatase inhibitor mixture (Sigma, MO). The equal amount of total protein was subjected to SDS–PAGE analysis, and immunoblotting with the appropriate antibodies.

2.6. Soft agar colony formation

5×10^3 cells were plated in 0.35% low melting point agarose/growth medium onto six-well plates with a 0.6% agarose underlay and incubated at 37 °C in humidified incubator for 14 days. The medium was changed every 3–4 d. At the end of the treatment, the cells were washed with PBS and incubated in a solution of 0.005% crystal violet and 10% formalin for 10 min and then rinsed with water. The number of colonies on each plate was determined using ImageJ software.

2.7. Wound-healing migration assay

SKVO-3 cells were starved to inactivate cell proliferation and then wounded by pipette tips. 10% FBS was added to DMEM cell culture medium. Images of the cells were taken after 16 h of incubation. Migrated cells were quantified manually. Three independent experiments were performed.

2.8. Transwell migration assay

A Transwell migration assay (BD Biosciences) with 6.5-mm-diameter polycarbonate filters (8-μm pore size) was used to study the invasion motility of the cells. In brief, the filter of the Transwell plate was coated with Matrix gel. The bottom chambers were filled with 500 μL of DMEM containing 10% FBS. Cells (4×10^4) suspended in 100 μL of DMEM containing 0.5% FBS were seeded in the top chambers. Cells were allowed to migrate for 24 h. Nonmigrated cells were removed with cotton swabs, and migrated cells were fixed with cold 4% paraformaldehyde and stained with 1% crystal violet. Images were taken using an inverted microscope (Olympus), and migrated cells were quantified by manual counting.

2.9. Immunofluorescence staining

Cells grown on a glass slide were fixed in 4% paraformaldehyde and permeabilized with 0.5% Triton X-100. Slides were washed with PBS and blocked with 5% bovine serum albumin (BSA) in PBS for 20 min at room temperature. Slides were then incubated overnight with primary antibodies (1:200) against vinculin in 1 × PBS with 5% BSA. Samples were washed with PBS and incubated with Alexa Fluor® 568 labeled goat anti-mouse secondary antibody (1:300) for 1 h. Next, samples were washed with PBS and incubated with Alexa Fluor® 488 Phalloidin (1:1000) for 20 min. The slides were then rinsed and mounted with DAPI (4, 6-diamidino-2-phenylindole) mounting solution. Images were analyzed with a Zeiss LSM510 microscope. The number of focal adhesions per cells were calculated based on the positive signal of vinculin (Red). Total 50 cells were counted and the results were averaged from three independent experiments.

2.10. Statistical analysis

All data were analyzed using the SPSS (version 20.0; IBM Corporation, Armonk, NY, USA) software program. P value less than 0.05 was regarded as statistically significant.

3. Results

3.1. Silence of TRPM7 in OVCA cells inhibited cell growth and colony formation

Previously, we have shown that TRPM7 highly expressed in ovarian carcinomas and overexpression of TRPM7 was significantly associated with poor prognosis in patients with ovarian cancer. In this study, the expression of TRPM7 was further examined in several human OVCA cell lines including SKOV-3, SW626 and PA-1. As shown in Fig. 1A, the expression levels of TRPM7 was elevated in all OVCA cells compared to normal ovarian tissue, which was similar to what we observed in ovarian carcinomas. In order to further study the roles of TRPM7 in OVCA cell lines, we then used a short hairpin RNA (shRNA) to specifically silence the expression of TRPM7 in OVCA cell lines. We found that one short hairpin RNA named

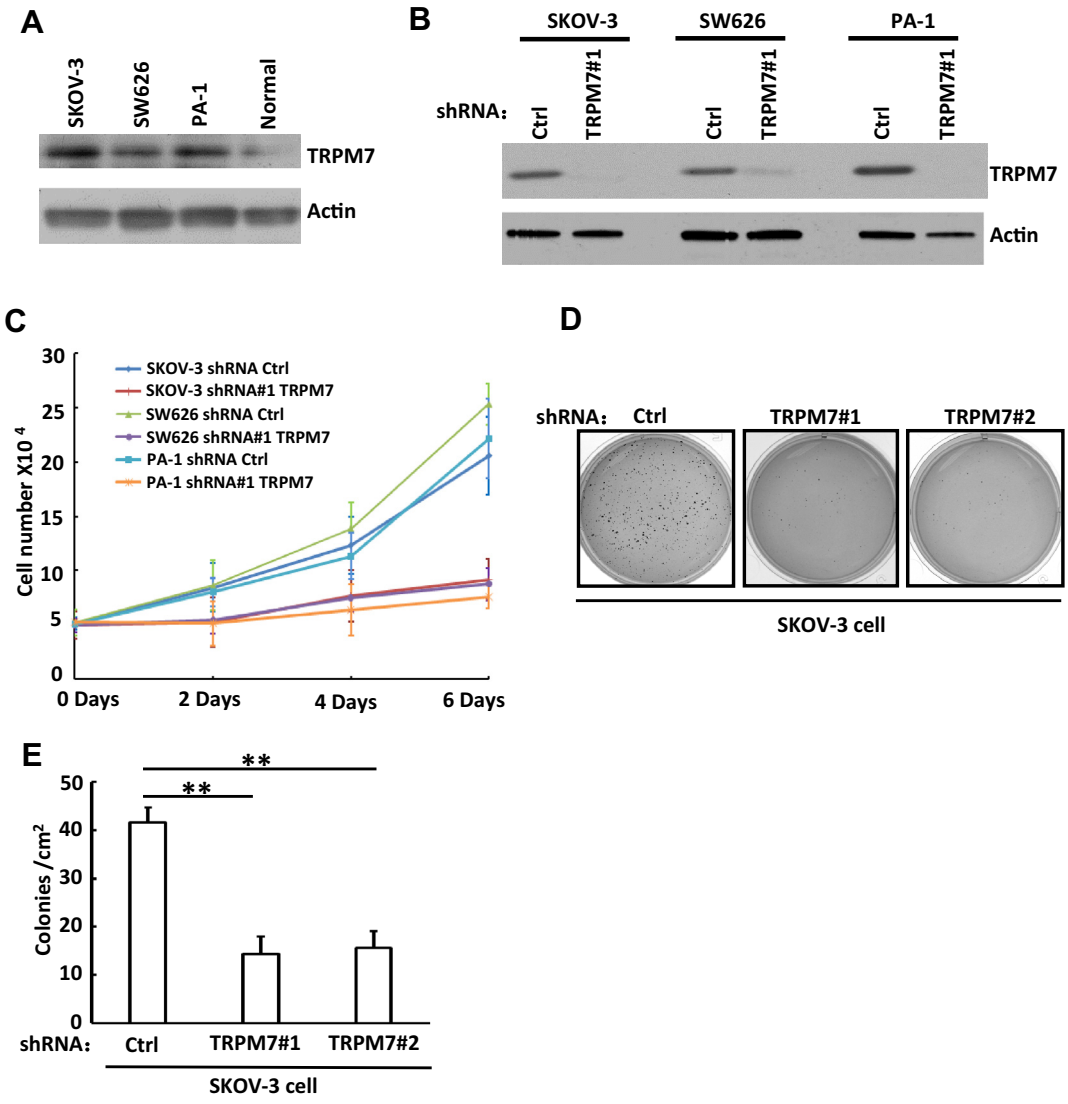

Fig. 1. Silence of TRPM7 in OVCA cells inhibits cell proliferation and colony formation. (A) Expression levels of TRPM7 in SKOV-1, SW626 and PA-1 cells were analyzed by Western blotting. (B) TRPM7 expression was examined by Western blotting in different OVCA cell lines expressing either control or TRPM7#1 shRNA. (C) Cell growth curve in OVCA cell lines expressing either control or TRPM7#1 shRNA. (D) Representative 35-mm plates of SKOV-3 cells expressing control, TRPM7#1 or TRPM7#2 shRNA cultured for 14 days. At the end of day, the cells were fixed and stained with crystal violet. (E) Quantitative data of colony formation assays. Colonies greater than 1 mm in diameter were counted. $^{**}P < 0.01$; relative to untreated cells. All data were representative results of three independent experiments.

TRPM7#1-shRNA successfully silenced expression of TRPM7 in SKOV-3, SW626 and PA-1 cells (Fig. 1B). Once TRPM7 silenced cell lines were established, we then asked whether silence of TRPM7 influence cell proliferation. As shown in Fig. 1C, we found that silence of TRPM7 significantly inhibited cell growth in all tested OVCA cell lines. Since cancer cells contain the ability to grow unattached to a surface, we then used a soft agar colony formation assay to further measure the effect of TRPM7 silencing on anchorage independent growth in OVCA cells. We found that silence of TRPM7 also significantly inhibited cell colony formation in SKOV-3 cells. Thus, these data demonstrate that silence of TRPM7 by shRNA inhibits cell proliferation and colony formation in OVCA cells.

3.2. Silence of TRPM7 in ovarian cancer cells inhibited cell migration and invasion

To investigate the effect of TRPM7 on cancer cell migration, we first performed wound-scratch assays to determine the migration ability of SKOV-3 cells after silencing TRPM7 by shRNA. As shown in Fig. 2A, the cells migrated more slowly after knocking down TRPM7 by two different of shRNA respectively and the number of migrating cell was also significantly decreased, compared to the control cells (Fig. 2B). Furthermore, in order to evaluate the effect of TRPM7 on cancer cell invasion, we then performed Matrixgel transwell assays in SKOV-3 cells. We found that the ability of SKOV-3 cells to invade into Matrixgel matrix was significantly decreased after silence of TRPM7 by these two different shRNAs respectively (Fig. 2C and D). Thus, these data suggest that TRPM7 plays important roles in mediating cell migration and invasion in OVCA cells.

3.3. TRPM7 regulates activation of multiple signaling pathways to mediate cell proliferation and migration

To study the molecular mechanisms by which TRPM7 mediate cell proliferation and migration in OVCA cells, the activities of several signaling molecules related to cell proliferation and migration were investigated by Western blotting. We found that silence of

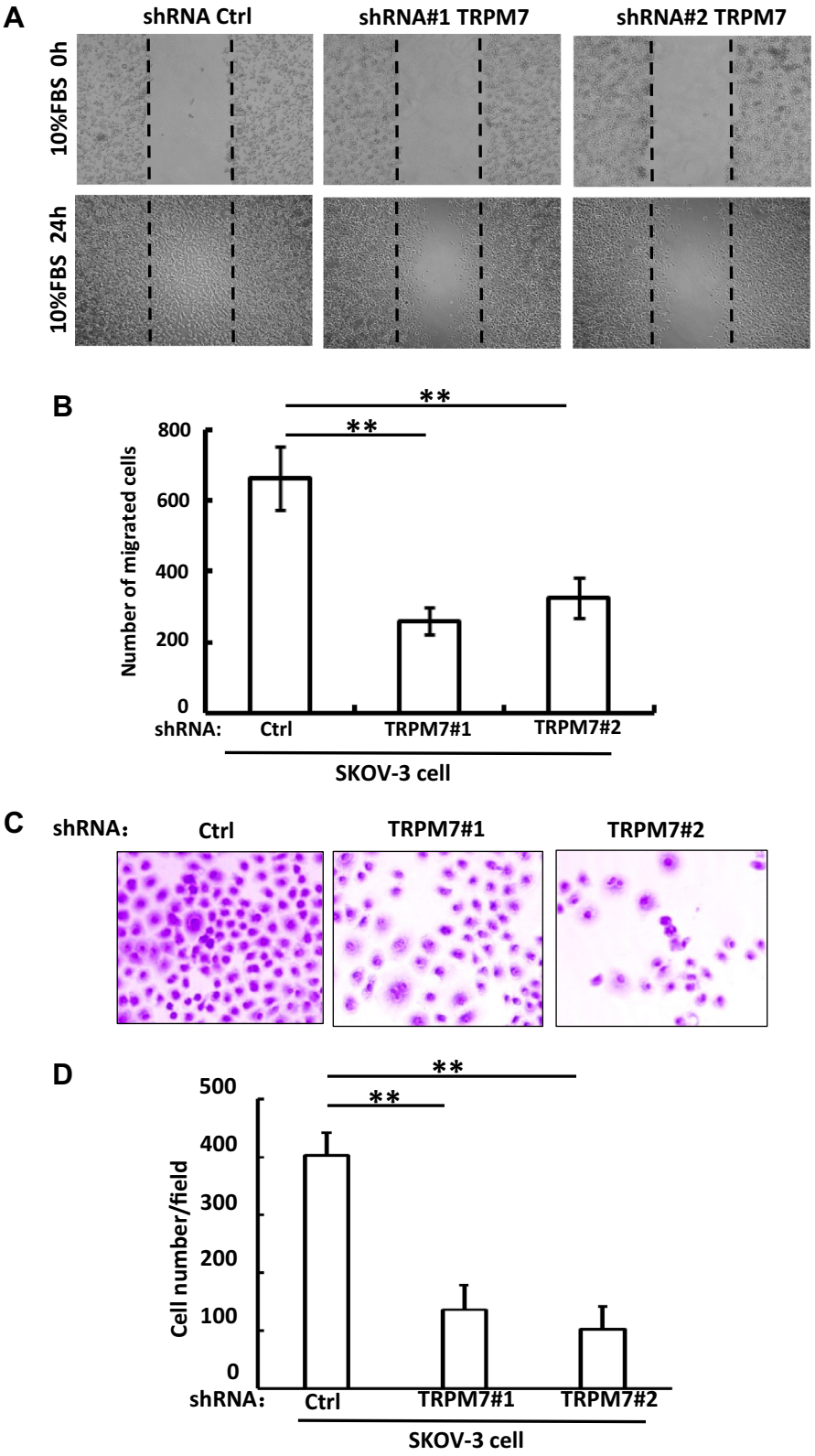

Fig. 2. Silence of TRPM7 in OVCA cells inhibits cell migration and invasion. (A) SKOV-3 cells expressing control, TRPM7#1 or TRPM7#2 shRNA were scratched by a pipette and cultured for 16 h. Representative images of wound-healing were shown. (B) Quantitative data of migrated cells in wound-healing assay. (C) SKOV-3 cells expressing control, TRPM7#1 or TRPM7#2 shRNA were scratched were seeded in Transwells covered with Matrixgel and cultured for 16 h. Representative images of transwell assay were shown. (D) Quantitative data of invasive cells in Matrixgel transwell assay. $^{**}P < 0.01$; relative to untreated cells. All data were representative results of three independent experiments.

TRPM7 significantly decreased phosphorylation levels of Akt, Src and p38 in OVCA cells, when compared to control shRNA (Fig. 3). As Akt, Src and p38 signaling pathways have been shown to play key roles in cell proliferation, motility and differentiation, these data suggest that these signaling pathways may be involved in TRPM7 mediated cell proliferation and migration in OVCA cells.

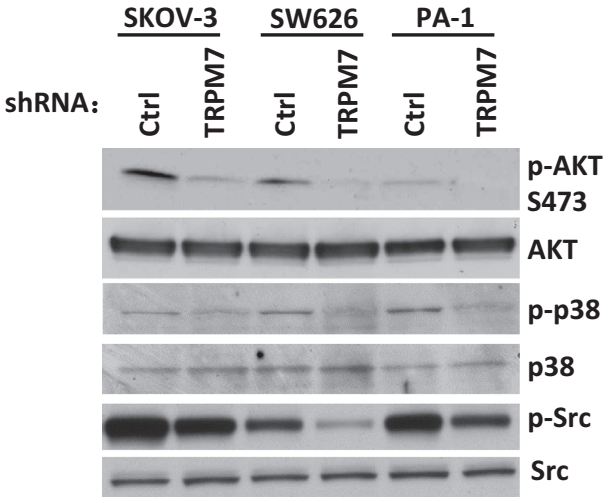

Fig. 3. TRPM7 regulates the phosphorylation of Akt, Src and p38 in OVCA cells. Phosphorylation of Akt, Src and p38 were analyzed by immunoblotting with phosphorylation-specific and control antibodies in SKOV-3, SW626 and PA-1 cells expressing either control or TRPM7#1 shRNA.

3.4. Silence of TRPM7 increases cell adhesion assembly

Cell migration and invasion are dynamically regulated by the formation and disassembly of focal adhesions. Since silence of TRPM7 inhibited cell migration and invasion, we then asked whether silence of TRPM7 influence the formation of focal adhesions. Though immunofluorescent staining of filamentous actin and focal adhesion marker vinculin in SKOV-3 cells, we found that silence of TRPM7 greatly increase the number of filamentous actin and focal adhesions (Fig. 4A and B). Thus, this data suggest that TRPM7 may manipulate adhesion dynamics to allow cell migration and invasion.

4. Discussion

TRPM7 is a nonselective cation channel with kinase activity. It has been shown to overexpress in various types of cancers and contribute to tumor development and progression [9]. Recently, we have identified that TRPM7 also highly expressed in ovarian carcinomas and its overexpression was associated with poor prognosis of the patients with OVCA [16]. However, the function of TRPM7 in ovarian cancer is mostly unknown. In this study, we

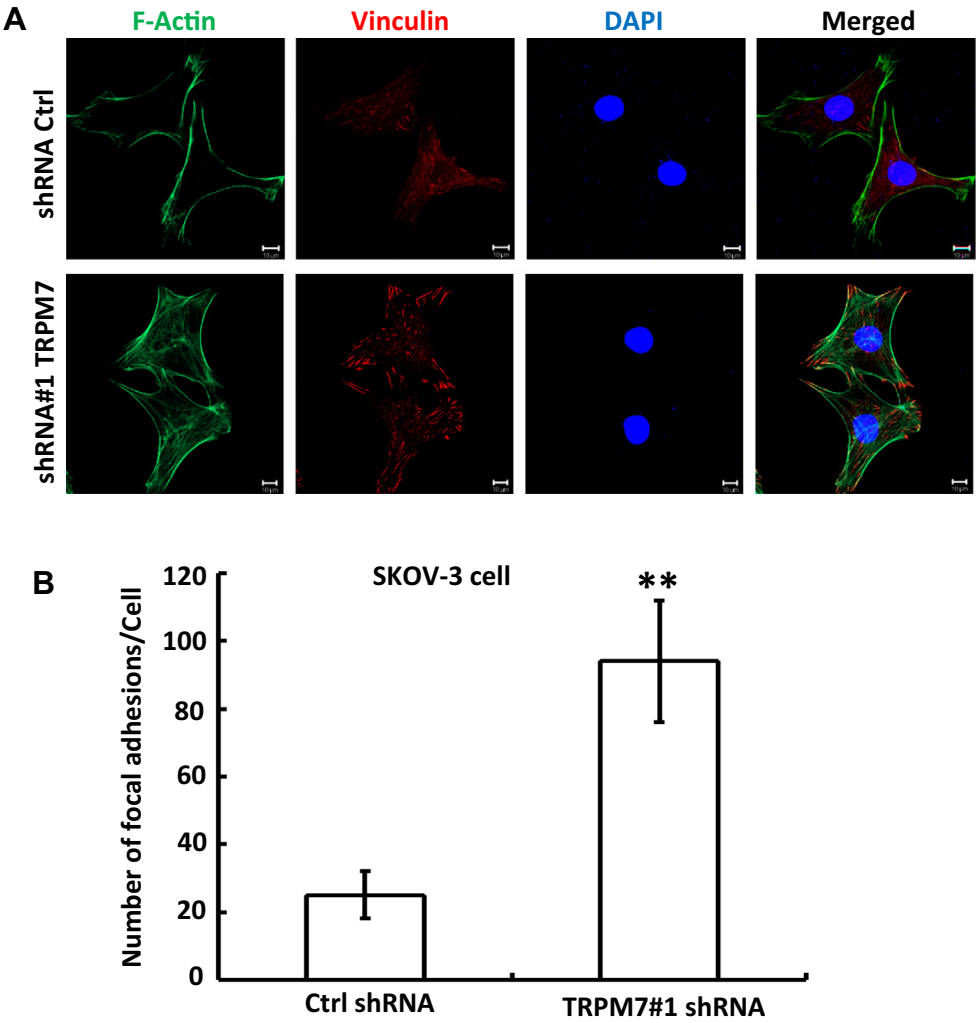

Fig. 4. Silence of TRPM7 increases cell adhesion assembly. (A) Immunofluorescence staining of SKOV-3 cells expressing control or TRPM7#1 shRNA with focal adhesion antibody anti-vinculin (Red) and Alexa488-phalloidin (Green) to visualize the actin cytoskeleton. Nuclear was stained with DAPI (Blue). (B) Quantitative data of focal adhesions in SKOV-3 cells expression control or TRPM7#1 shRNA. $^{**}P < 0.01$; relative to untreated cells. All data were representative results of three independent experiments. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)

confirmed that TRPM7 overexpressed in several ovarian cancer cell lines and further found that silence of TRPM7 by shRNA significantly inhibited cell proliferation and colony formation. These results are similar to recent studies that depressing the function of TRPM7 by RNA interference inhibited cancer cell growth [13,17]. Furthermore, it has been demonstrated that pharmaceutical inhibition of TRPM7 activity suppressed several types of cancer cell growth and proliferation including breast cancer, pancreatic cancer and hypopharyngeal squamous cell carcinoma cells [14,15,18]. Thus, our data suggests that TRPM7 may serve as a novel pharmaceutical target in ovarian cancer therapy.

Our previous study indicated that higher expression levels of TRPM7 in ovarian carcinomas was significantly associated with pelvic metastasis in patients with OVCA [16]. In this study, we further demonstrated that silence of TRPM7 inhibited migration and invasion in OVCA cells, suggesting an important role of TRPM7 involved in OVCA metastasis. It has been reported that TRPM7 regulated migration and invasion in other types of cancer cells including breast cancer, lung cancer, pancreatic cancer and gastric cancer cells. Since cancer cell migration and invasion is regulated by multiple signal-transduction pathways, we further explored the activities of several signal molecules involved in ovarian cancer migration and invasion. We found that the phosphorylation levels of Akt, Src and p38 were significantly decrease after silence of TRPM7 by shRNA. Previous studies have shown that these key molecules in signal-transduction pathways were critical for ovarian cancer progression by promoting tumor cell proliferation, migration and invasion. For example, the aberrantly activation of Akt signaling pathway has been observed in OVCA and more than 36% of the patients with OVCA contain PI3K gene amplification [19]. The activation of Akt is critical for OVCA progression and metastasis. Currently, several inhibitors for PI3K or Akt have been developed for OVCA therapy [20]. The Src protein is an non-receptor tyrosine kinase. The aberrantly activation of Src kinase is related to tumor invasion and metastasis in multiple types of cancers [21]. It has been reported that Src kinase was aberrantly active in OVCA and contribute to tumor invasion, metastasis and chemo-resistance [22,23]. Thus, our data suggest that TRPM7 modulating migration and invasion of OVCA cells is associated with Akt, Src and p38 signaling pathways, which are critical for OVCA development and progression. Previous studies demonstrated that silence of TRPM7 enhance cell adhesion and increase the number of peripheral adhesion complexes [11]. As high capability of cell adhesion retards cell migration and invasion, we also observed that silence of TRPM7 in SKOV-3 cells increased filamentous actin and the number of focal adhesions. Since Akt and Src signaling pathways are involved in regulation of cell adhesion, these events may function as downstream effects of the signaling pathways influenced by TRPM7 silencing. Nevertheless, the precise mechanisms how TRPM7 coordinately regulates these events to mediate cell migration and invasion needs to further systemic studies.

As a calcium permeable ion channel, TRPM7 is involved in calcium influx in both physiological and pathological conditions. Previous studies show that TRPM7 can modulates cell migration through directly regulating calcium flickers [24]. Moreover, the change of intracellular calcium level influences the activities of many signaling transduction pathways including Akt, Src and p38 in normal and cancer cells [25–27]. Thus, it would be expected that TRPM7 mediated calcium influx may play an important role involved in cell proliferation, migration and invasion in OVCA cells. Our future study need to test this interesting hypothesis in OVCA cells.

Taken together, our present study demonstrated that silence of TRPM7 by shRNA inhibited proliferation, migration and invasion of OVCA cells. We propose that TRPM7 mediates cell proliferation, migration and invasion at least in part involving in Akt, Src and p38 signaling pathways and the formation of cell adhesion complexes in OVCA cells.

Disclosure

The authors report no conflicts of interest in this work.

Acknowledgments

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