

BDNF activates TrkB/PLC γ 1 signaling pathway to promote proliferation and invasion of ovarian cancer cells through inhibition of apoptosis

Y. XU¹, W.-G. JIANG², H.-C. WANG¹, T. MARTIN², Y.-X. ZENG³, J. ZHANG³ Y.-S. QI¹

¹Department of Gynaecology, Zibo Maternity and Child Health Hospital, Zibo, China

²Cardiff China Medical Research Collaborative (CCMRC), Cardiff University School of Medicine, Henry Wellcome Building, Heath Park, Cardiff, UK

³PLA Navy Qingdao special service recuperation Center, Qingdao, China

Abstract. – OBJECTIVE: Abnormal expression and activation of tropomyosin-related kinase receptor B (TrkB) are observed in many pathological conditions, including many types of cancer. We try to explore the relationship between ovarian cancer and Brain-derived neurotrophic factor (BDNF), a ligand of TrkB.

MATERIALS AND METHODS: Human ovarian cancer cell line SKOV-3 was used in this study. qPCR, immunohistochemistry, and immunoblot were used to assay BDNF and TrkB expression level. Scratch assay was used to test the cell motility, and transwell assay was used to test the cell migration ability.

RESULTS: We found that BDNF promotes the proliferation and invasion of human ovarian cancer SKOV-3 cells depend on the activation of TrkB. To illuminate the downstream pathway of BDNF/TrkB, we silenced AKT1 and PLC γ 1 by siRNA. The functional assay showed that activated PLC γ 1 signaling pathway is necessary for the proliferation and invasion of cancer cells other than the AKT pathway. Further study showed that PLC γ 1 could inhibit the apoptosis of cancer cells.

CONCLUSIONS: BDNF triggers TrkB/PLC γ 1 signaling pathway to promote proliferation and invasion of ovarian cancer cells through inhibition of apoptosis.

Key Words

BDNF, TrkB, PLC γ 1, Ovarian cancer, Proliferation, Invasion, Apoptosis.

Introduction

Ovarian cancer is the third most common type of cancer and the fourth leading cause of cancer-related death worldwide^{1,2}. But the mechanism by which cancer develops still remains poorly understood. It

is well known that growth factors and hormones are very important during the development of the ovary and other organs. Overexpression of growth factors and hormones, and abnormal expression or activation of their receptors may regulate the progression of many types of cancer including ovarian cancer^{3,4}.

Brain-derived neurotrophic factor (BDNF) is a very important nerve growth factor and its binding and subsequent activation of its receptor, Tropomyosin-related kinase B (TrkB) plays a critical role in the development of nervous system⁵. More and more researches show that BDNF is a multi-functional growth factor that exerts various effects on some non-neuronal cells and plays a role in the progression of pathological diseases⁶. Recently, the function of BDNF and TrkB is uncovered in many types of cancer such as breast cancer⁷.

TrkB is a tyrosine kinase receptor which binds BDNF and is regarded as a potential target in several cancers. TrkB activates a couple of intracellular signaling pathways to regulate cell proliferation, differentiation, invasiveness, and angiogenesis⁸⁻¹⁰.

We showed activation of BDNF/TrkB signaling pathway could promote cell proliferation and invasion of ovarian cancer cells *in vitro*. We demonstrated that expression of TrkB increased in the ovarian cancer tissues by immunohistochemistry. BDNF promotes the activation of TrkB in ovarian cancer cell line SKOV-3 as revealed by Western blot. Further results suggested that silence of TrkB resulted in a significant inhibition of cell proliferation and invasion induced by BDNF. By inhibition of downstream gene AKT1 and PLC γ 1, we found that only silence of PLC γ 1 blocked the effect of BDNF/TrkB. We finally showed that inhibition of TrkB or PLC γ 1 promotes apoptosis of SKOV-3.

Materials and Methods

Cell Culture

Human ovarian cancer cell line SKOV-3 (ATCC® HTB-77™) was purchased from American Type Culture Collection (ATCC, Manassas, VA, USA) and cultured in DMEM with 10% fetal bovine serum (Invitrogen, Carlsbad, CA, USA) supplemented with penicillin-streptomycin (1:100; Invitrogen, Carlsbad, CA, USA). All the cells were cultured in a humidified atmosphere of 5% CO₂ at 37°C.

For BDNF treatment, cells were seeded and treated with recombinant human BDNF (Biorbyt Ltd, Cambridge, UK; Orb80270, 10 µg, Lot#: C9154) in FBS free medium at a dose of 0, 50, 100 ng/ml, respectively. PBS was added as control. 48 h after treatment, cells were analyzed as indicated.

Western Blotting

30 µg proteins were denatured at 95°C for 5 min and loaded into each lane of 4%-12% polyacrylamide gels and then transferred onto nitrocellulose membranes (Bio-Rad, Hercules, CA, USA) at 100 V for 60 min. The membrane was blocked with 5% non-fat dry milk in Tris-Buffered Saline and Tween (TBST) for 1 h at RT with shaking. Then, we incubated it with anti-phosphor-TrkB (pTrkB, 1:1000), anti-total-TrkB (TrkB, 1:1000; Santa Cruz Biotechnology, Santa Cruz, CA, USA), anti-phosphor-AKT (pAKT1, 1:1000) or anti-total-AKT (AKT1, 1:1000) antibodies (Proteintech, Rosemont, IL, USA) for 16 h at 4°C. Then, we washed it 3 times for 10 min with TBST, we incubated it with secondary antibodies conjugated with horseradish peroxidase (HRP) (1:10000, Proteintech, Rosemont, IL, USA) for 1 h. Detection was performed using the ECL kit (Pierce, Rockford, IL, USA). The β-actin signal was used as a loading control.

Immunohistochemistry

The samples were fixed in 4% paraformaldehyde (4% PFA) for 16 h and then embedded in paraffin. Immunohistochemical study of BDNF and TrkB protein expression was performed as following protocol. 5 µm sections were dewaxed in xylene twice for 10 min and rehydrated sequentially in (100% ethanol twice for 1 min, 95% ethanol twice for 1 min, 75% ethanol twice for 1 min and ddH₂O 1 min) and antigen retrieval was performed by heating the sections at 98°C with 10 mM sodium citrate buffer pH6.0 for 15 min. Next, we added 3% H₂O₂ for 15 min to block endog-

enous peroxidase activity and followed by 10% goat serum blocking for 1 h at room temperature. Primary antibody (anti-TrkB 1:80, anti-BDNF 1:60; Abcam, Cambridge, MA, USA) was added and incubated at 4°C overnight. The expression was detected by DAB (Zhongshan Jinqiao Beijing). Sections were counterstained with hematoxylin and mounted.

siRNA Transfection

To knockdown TrkB, AKT1 and PLCγ1, scramble control, siTrkB, siAKT1 and siPLCγ1 siRNA were purchased from Santa Cruz Biotechnology (Santa Cruz, CA, USA). Transfection of 10 µM siRNA in RNAi Max lipid reagent (Invitrogen, Carlsbad, CA, USA) was performed in SKOV-3 cells according to the manufacturer's instruction. 48 h after transfection cells were harvested for further study.

Cell Proliferation Assay

Cell Proliferation was determined by direct cell count. Cells were seeded in 24-well plates at low density (1x10⁴) and allowed to attach overnight. Then cells were treated with indicated reagents and then cultured for 5 days. After 5 days' culture, cells were detached by trypsin/EDTA and counted in a hemocytometer.

Scratch Assay

In vitro scratch assay was used to assess cell motility. Cells were allowed to grow to confluence. Then a scratch was made in the cell layer with a sterile micropipette tip. The cell layer was washed twice with culture media and incubated for 24 h. The size of the scratch was measured at 4 random sites.

Cell Migration and Invasion Assay

Cell migration assays were performed using modified Boyden chambers with 8 µm-pore polycarbonate membrane (Corning, Corning, NY, USA). Cells (5 x10⁵ in 100 µl) in serum-free medium were seeded in the upper compartment coated with 0.1% gelatin of the chamber or Matrigel (1:10 dilution in medium) for invasion assay. BDNF in 700 µl normal medium was added in lower wells as specified. Cells were allowed to migrate for 48 h at 37°C in 5% CO₂. Cells on the lower side of the membrane were fixed with 4% PFA, stained with 1% crystal violet and counted under bright field under high power field (100x). For each triplicate, the number of cells in ten random fields was determined, averaged counts were recorded.

TUNEL Assay

TUNEL assay was performed using the TUNEL kit (Roche Diagnostics, Mannheim, Germany) according to the manufacturer's instructions. Briefly, cells were fixed with 4% paraformaldehyde in PBS, permeated with 0.1% Triton X-100 in PBS and incubated with 50 μ l TUNEL reaction mixture for 1 h at 37°C. The reaction was terminated by washing with 1 x PBS. Cells were counted in 5 randomly-chosen different fields under a microscope, total 500 cells were counted, and the percentage of apoptotic cells was quantified.

Statistical Analysis

SPSS Statistical Package version 16 (SPSS Inc., Chicago, IL, USA) was used for statistical analysis and expressed as means SE. Independent two group's analysis were made using the Student's *t*-test. $p < 0.05$ was considered statistically significant.

Results

Highly Expression of BDNF and TrkB in Ovarian Cancer with Poor Prognosis

To study the expression and localization of BDNF and TrkB in ovarian cancer tissue, we performed immunohistochemistry, the result showed BDNF and TrkB localized at the cytoplasm and cell membrane of epithelial cells (Figure 1). Among the different diagnostic groups,

the normal ovary tissue was negative for BDNF and TrkB and all the invasive cancers and the metastatic foci were positive. Protein expression of BDNF and TrkB were significantly higher in invasive cancer and metastatic foci when compared with normal tissues. So BDNF and TrkB may play a role in the progression of ovarian carcinogenesis.

BDNF Promotes Proliferation and Migration and Invasion of Ovarian Cancer Cells

To assess the function of BDNF in the proliferation of ovarian cancer, we treated SKOV-3 cells with recombinant human BDNF, the results showed that BDNF could promote the proliferation of cancer cells (Figure 2A). We then studied the role of BDNF in controlling the migration and invasion of ovarian cancer cells. Using scratch wound healing assay, we showed that treatment of BDNF resulted in a significant upregulation of the migration capacity of SKOV-3 cells (Figure 2B). Consistent with the scratch assay, transwell assay also showed a significant increase of cells migrated to the lower side of the membrane (Figure 2C). To assess the function of BDNF in the invasion ability of ovarian cancer, invasion of the Matrigel-coated transwell assay was performed. The result showed that BDNF promoted invasion of SKOV-3 cells significantly (Figure 2D). These results demonstrated that BDNF could promote proliferation and invasion capacity of ovarian cancer cells.

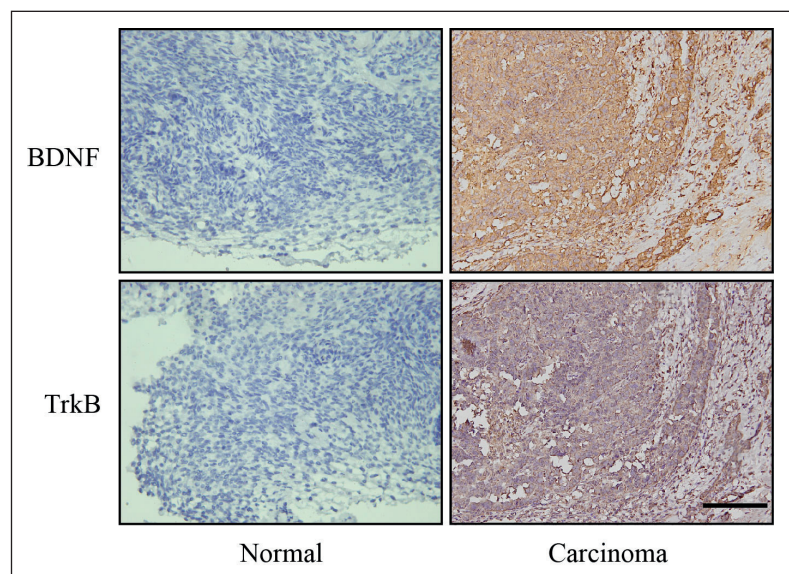


Figure 1. Highly expression of BDNF and TrkB in ovarian cancer with poor prognosis. Immunohistochemistry showed expression of BDNF and TrkB were absent in epithelium of normal ovary tissue but highly present in ovarian carcinomas. Representative pictures were shown.

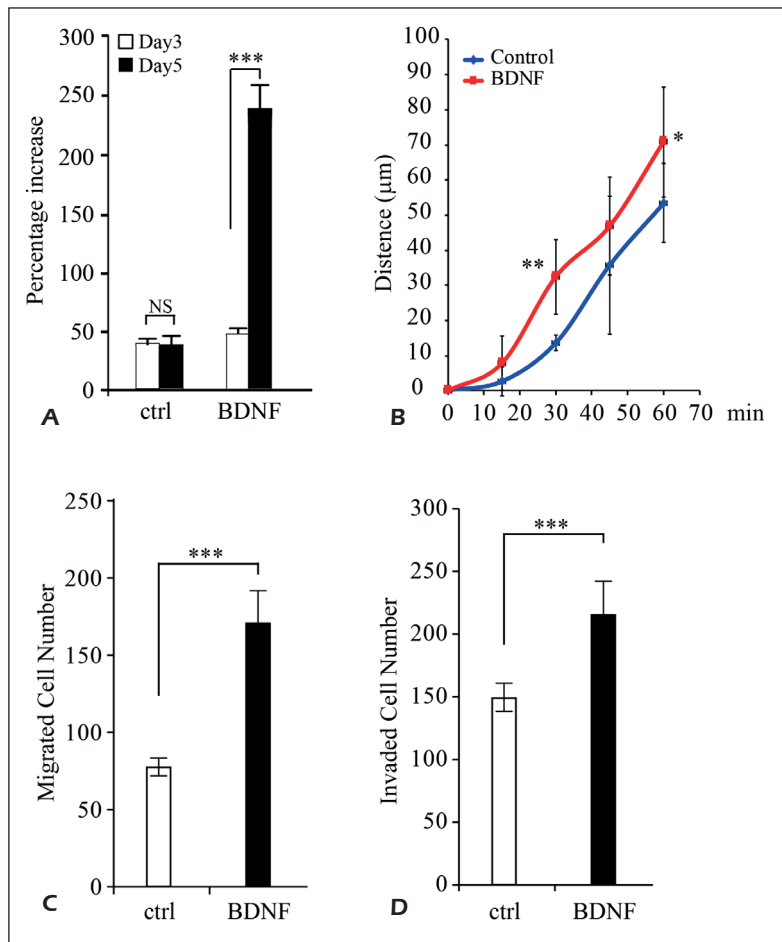


Figure 2. BDNF promotes proliferation and migration and invasion of ovarian cancer cells. **A**, Cells were treated with BDNF (50 ng/ml) for 48 h, the proliferation of SKOV-3 cells increased significantly after 5 days. Scratch assay showed higher motility of SKOV-3 cells after BDNF treatment (**B**), transwell migration (**C**) and invasion (**D**) assay suggested BDNF enhanced the migration and invasion of SKOV-3 cells. * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$.

BDNF Upregulates Expression and Phosphorylation of TrkB and Enhances Cell Proliferation and Migration and Invasion

To gain further insight into the mechanism by which BDNF promotes the development of ovarian cancer, expression and phosphorylation of TrkB under treatment of BDNF were detected. Western blot of TrkB and phosphor-TrkB showed treatment of BDNF increased the expression and phosphorylation of TrkB significantly in SKOV-3 cells (Figure 3A). Then, we silenced the expression of TrkB by siRNA, and proliferation, migration and invasion assay were performed. The results showed without TrkB expression, BDNF couldn't enhance cell proliferation and migration and invasion (Figure 3B, 3C and 3D).

TrkB/PI3K Signaling Pathway Is Not Necessary to Promote Proliferation and Migration of Ovarian Cancer Cells

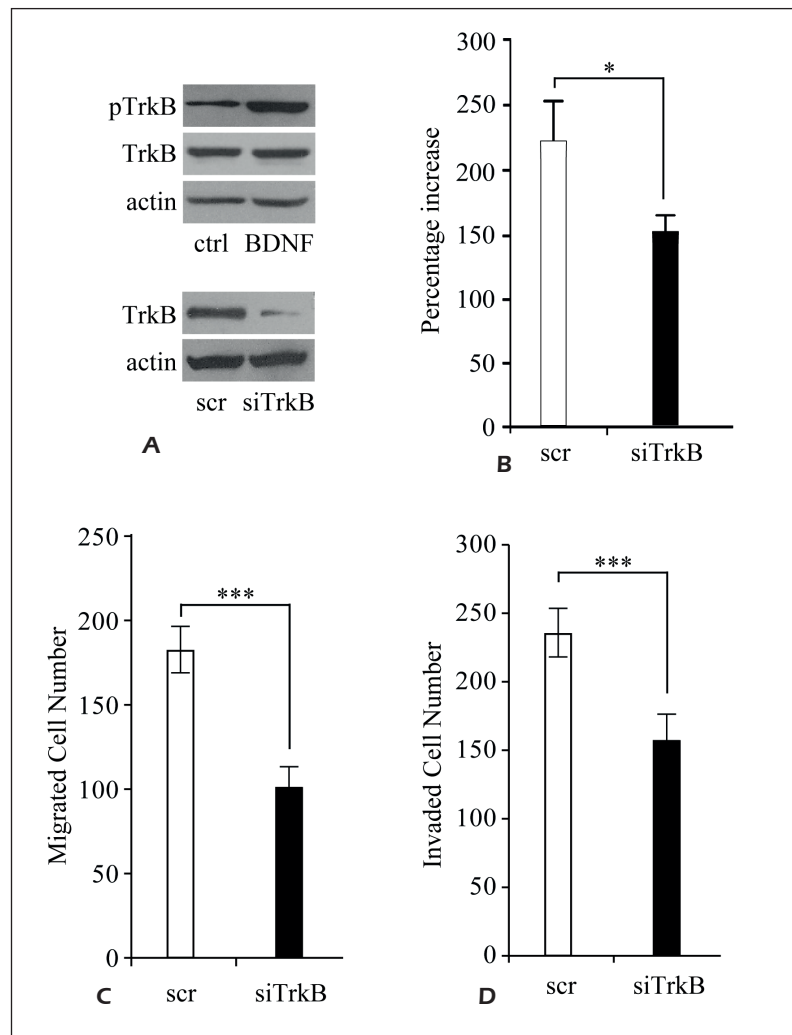
It has been reported that one of the downstream pathways of BDNF/TrkB is PI3K-AKT

pathway. To study whether this pathway regulates cell proliferation and migration in ovarian cancer, we silenced AKT1 by siRNA (Figure 4A), proliferation assay showed no difference with scramble control group (Figure 4B). Invasion assays were performed, the result showed that knockdown of AKT1 showed no difference of migration ability of SKOV-3 cells (Figure 4C).

TrkB/PLC γ 1 Signaling Pathway Is Necessary to Promote Proliferation and Migration of Ovarian Cancer Cells

It has been reported that one of the downstream pathways of BDNF/TrkB is PLC γ 1 pathway. To study whether this pathway regulates cell proliferation and migration in ovarian cancer, we silenced PLC γ 1 by siRNA (Figure 5A), proliferation assay showed that, compared with scramble control group, BDNF couldn't increase cell proliferation without PLC γ 1 (Figure 5B). Invasion assays showed that knockdown of PLC γ 1 significantly decreased cell migration ability of SKOV-3 cells (Figure 5C).

Figure 3. BDNF upregulates expression and phosphorylation of TrkB and enhances cell proliferation and migration and invasion. **A**, Treatment of BDNF recombinant protein promoted expression and activation of TrkB by Western blot. Silencing of TrkB by siTrkB (A) significantly abolished effects of BDNF on proliferation (**B**) and migration (**C**) and invasion (**D**) of SKOV-3 cells. * $p < 0.05$, ** $p < 0.01$.



TrkB/PLC γ 1 Signaling Pathway Inhibits Cell Apoptosis to Promote Proliferation and Migration of Ovarian Cancer Cells

To demonstrate the mechanism of TrkB/PLC γ 1 signaling pathway, we detected the cell apoptosis in SKOV-3 cells. TUNEL assay was performed and the result showed that silencing of TrkB enhanced apoptosis of SKOV-3 cells. Further, silencing of PLC γ 1 by siRNA was sufficient to enhanced apoptosis of SKOV-3 cells (Figure 6A and 6B).

To summarize, our results demonstrated that BDNF-mediated progression of ovarian cancer is mediated by TrkB/PLC γ 1 signaling pathway.

Discussion

Overexpression of growth factors and hormones and abnormal expression or activation of

their receptors may regulate the progression of many types of cancer including ovarian cancer. In this study, we investigated the role of the BDNF/TrkB pathway in ovarian carcinogenesis. Immunohistochemistry results showed increased BDNF and TrkB expression in ovarian cancers when compared with the normal ovary. This suggested that dysregulated TrkB expression was critical in ovarian carcinogenesis. More importantly, high TrkB expression level was associated with poor patient outcome and was an independent prognostic factor^{11,12}.

BDNF could bind with TrkB and activate the downstream signaling pathway. It has been documented that an autocrine loop existed between TrkB and BDNF in malignant tumors such as neuroblastoma and myeloma^{12,13}. In the autocrine loop, high expression of BDNF induced expression of TrkB and *vice versa*. We found that both BDNF and TrkB were expressed in cancer cells

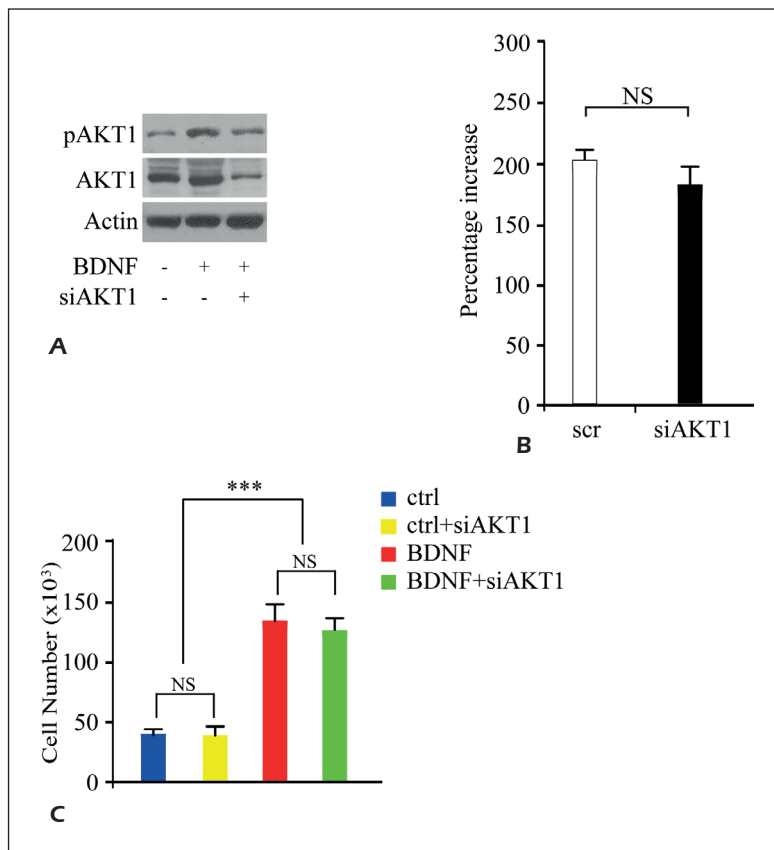
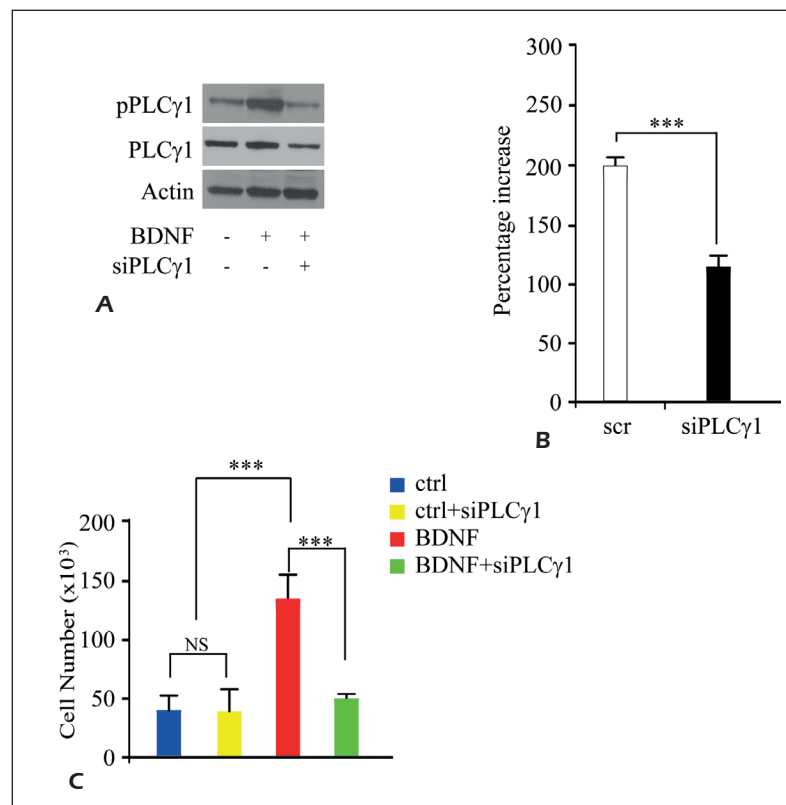


Figure 4. TrkB/PI3K signaling pathway is not necessary to promote proliferation and migration of ovarian cancer cells. A, Treatment of BDNF recombinant protein promoted phosphorylation of AKT1 by Western blot. Silencing of AKT1 by siAKT1 (A) showed no effects of BDNF on proliferation (B) and invasion (C) of SKOV-3 cells. *** $p < 0.001$.

Figure 5. TrkB/PLC γ 1 signaling pathway is necessary to promote proliferation and migration of ovarian cancer cells. A, Treatment of BDNF recombinant protein promoted phosphorylation of PLC γ 1 by Western blot. Silencing of PLC γ 1 by siPLC γ 1 (A) significantly abolished effects of BDNF on proliferation (B) and invasion (C) of SKOV-3 cells. * $p < 0.05$, *** $p < 0.001$.



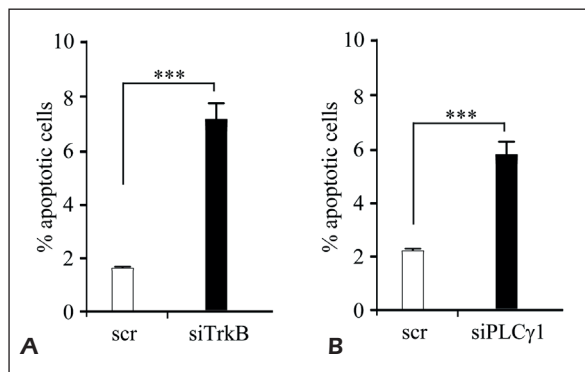


Figure 6. TrkB/PLCγ1 signaling pathway inhibits cell apoptosis to promote proliferation and migration of ovarian cancer cells. **A-B**, Silencing of TrkB and PLCγ1 by siTrkB and siPLCγ1 respectively significantly increased apoptosis of SKOV-3 cells. * $p < 0.05$.

and BDNF could activate TrkB on cancer cells and contribute to cancer progression. However, the mechanisms by which TrkB expression is induced in ovarian tumors was still not clear. We then demonstrated that PLCγ1 other than AKT1 is the necessary downstream effector of BDNF/TrkB signaling pathway. When PLCγ1 was silenced, BDNF induced cell proliferation and migration and invasion were hindered.

Several growth factors could activate PLCγ1 and PLCγ1 is required for cell proliferation and motility and invasion in different cell types^{14,15}. Strong evidence demonstrated that PLCγ1 is a critical enzyme in the development and maintenance of tumor metastasis. The role of PLCγ1 in proliferation was first identified in the study of the growth factor signaling, such as through EGFR and platelet-derived growth factor receptor (PDGFR) pathways. And a couple of studies have implicated PLCγ1 plays a critical role in the regulation of cancer cell invasion and metastasis via multiple mechanisms¹⁵. PLCγ1 is thought to regulate both cytoskeletal changes and migration associated with the metastatic process¹⁴⁻¹⁶. Here we showed PLCγ1 could inhibit apoptosis of cancer cells.

Conclusions

We showed here that the overexpression of TrkB predicts poor prognosis of ovarian cancer patients. Then, we demonstrated that BDNF/TrkB pathway promotes proliferation and invasion of ovarian cancer cell line SKOV-3. Furthermore, we reported PLCγ1 is the possible downstream

effector of TrkB signaling pathways in promoting ovarian cancer proliferation and invasion by inhibition of apoptosis which worth further research as potential therapeutic targets.

Conflict of Interests

The authors declare that there are no conflicts of interest.

References

- GILBERT L, BASSO O, SAMPALIS J, KARP I, MARTINS C, FENG J, PIEDIMONTE S, QUINTAL L, RAMANAKUMAR AV, TAKEFMAN J, GRIGORIE MS, ARTHO G, KRISHNAMURTHY S. Assessment of symptomatic women for early diagnosis of ovarian cancer: results from the prospective DOVe pilot project. *Lancet Oncol* 2012; 13: 285-291.
- JEMAL A, BRAY F, CENTER MM, FERLAY J, WARD E, FORMAN D. Global cancer statistics. *CA Cancer J Clin* 2011; 61: 69-90.
- WONG AS, LEUNG PC. Role of endocrine and growth factors on the ovarian surface epithelium. *J Obstet Gynaecol Res* 2007; 33: 3-16.
- YU X, LIU L, CAI B, HE Y, WAN X. Suppression of anoikis by the neurotrophic receptor TrkB in human ovarian cancer. *Cancer Sci* 2008; 99: 543-552.
- SEGAL RA, GREENBERG ME. Intracellular signaling pathways activated by neurotrophic factors. *Annu Rev Neurosci* 1996; 19: 463-489.
- ASGHARZADEH S, PIQUE-REGI R, SPOSTO R, WANG H, YANG Y, SHIMADA H, MATTHAY K, BUCKLEY J, ORTEGA A, SEEGER RC. Prognostic significance of gene expression profiles of metastatic neuroblastomas lacking MYCN gene amplification. *J Natl Cancer Inst* 2006; 98: 1193-1203.
- ROESLER R, DE FARIAS CB, ABUJAMRA AL, BRUNETTO AL, SCHWARTSMANN G. BDNF/TrkB signaling as an anti-tumor target. *Expert Rev Anticancer Ther* 2011; 11: 1473-1475.
- PEREZ-PINERA P, HERNANDEZ T, GARCÍA-SUÁREZ O, DE CARLOS F, GERMANA A, DEL VALLE M, ASTUDILLO A, VEGA JA. The Trk tyrosine kinase inhibitor K252a regulates growth of lung adenocarcinomas. *Mol Cell Biochem* 2007; 295: 19-26.
- AU CW, SIU MK, LIAO X, WONG ES, NGAN HY, TAM KF, CHAN DC, CHAN OK, CHEUNG AN, PATANI N, JIANG WG, MOKBEL K. Tyrosine kinase B receptor and BDNF expression in ovarian cancers - effect on cell migration, angiogenesis and clinical outcome. *Cancer Lett* 2009; 281: 151-161.
- PATANI N, JIANG WG, MOKBEL K. Brain-derived neurotrophic factor expression predicts adverse pathological & clinical outcomes in human breast cancer. *Cancer Cell International* 2011; 11: 23.
- NAKAGAWARA A, AZAR CG, SCAVARDA NJ, BRODEUR GM. Expression and function of TRK-B and BDNF in human neuroblastomas. *Mol Cell Biol* 1994; 14: 759-767.
- PEARSE RN, SWENDEMAN SL, LI Y, RAFII D, HEMPSTEAD BL. A neurotrophin axis in myeloma: TrkB and BDNF promote tumor-cell survival. *Blood* 2005; 105: 4429-4436.

- 13) BRODEUR GM. Neuroblastoma: biological insights into a clinical enigma. *Nat Rev Cancer* 2003; 3: 203-216.
- 14) WELLS A, GRANDIS JR. Phospholipase C-gamma1 in tumor progression. *Clin Exp Metastasis* 2003; 20: 285-290.
- 15) PARK JB, LEE CS, JANG JH, GHIM J, KIM YJ, YOU S, HWANG D, SUH PG, RYU SH. Phospholipase signalling networks in cancer. *Nat Rev Cancer* 2012; 12: 782-792.
- 16) YAMAGUCHI H, CONDEELIS J. Regulation of the actin cytoskeleton in cancer cell migration and invasion. *Biochim Biophys Acta* 2007; 1773: 642-652.