Molecules and Cells

TRIB2 Stimulates Cancer Stem-Like Properties through Activating the AKT-GSK3β-β-Catenin Signaling Axis

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https://doi.org/10.14348/molcells.2021.0030
www.molcells.org

Tribbles homolog 2 (TRIB2) is implicated in tumorigenesis and drug resistance in various types of cancers. However, the role of TRIB2 in the regulation of tumorigenesis and drug resistance of cancer stem cells (CSCs) is still elusive. In the present study, we showed increased expression of TRIB2 in spheroid-forming and aldehyde dehydrogenase-positive CSC populations of A2780 epithelial ovarian cancer cells. Short hairpin RNA-mediated silencing of TRIB2 expression attenuates the spheroid-forming, migratory, tumorigenic, and drug-resistant properties of A2780 cells, whereas overexpression of TRIB2 increases the CSC-like characteristics. TRIB2 overexpression induced GSK3β inactivation by augmenting AKT-dependent phosphorylation of GSK3β at Ser9, followed by increasing β-catenin level via reducing the GSK3β-mediated phosphorylation of β-catenin. Treatment of TRIB2-ovexpressed A2780 cells with the phosphoinositide-3-kinase inhibitor LY294002 abrogated TRIB2-stimulated proliferation, migration, drug resistance of A2780 cells. These results suggest a critical role for TRIB2 in the regulation of CSC-like properties by increasing the stability of β-catenin protein via the AKT-GSK3β-dependent pathways.

Keywords: cancer stem cells, chemoresistance, ovarian cancer, stemness

INTRODUCTION

Ovarian cancer is a fatal disease among all gynecological cancers (Liao et al., 2014). Because ovarian cancers are asymptomatic or manifest vague symptoms at early clinical stages, most patients with ovarian cancer are diagnosed at advanced stages (Cannistra, 2004). For women diagnosed with advanced-stage disease, presenting with intraperitoneal metastasis, the 5-year survival rate is only 29% (Conic et al., 2011; Jones et al., 2017). Therefore, it is vital to understand the mechanism underlying metastasis and the development of resistance against chemotherapy in ovarian cancer to improve the clinical outcomes. Accumulating evidence suggests that ovarian cancer contains cancer stem cells (CSCs) or cancer stem-like cells possessing high tumor-initiating potential (Al-Alem et al., 2019). CSCs express high levels of stemness markers, such as OCT4, SOX2, and NANOG, and several CSC markers, including ALDH1, CD44, or CD133

Received 3 February, 2021; revised 11 April, 2021; accepted 12 May, 2021; published online 28 July, 2021

eISSN: 0219-1032

(Lupia and Cavallaro, 2017). CSCs exhibit spheroid-forming ability in three-dimensional (3D) suspension culture and have increased tumorigenesis, metastasis, and drug resistance potential. CSCs also exhibit epithelial to mesenchymal transition (EMT), which enhances motility and invasiveness of cancer cells (Lamouille et al., 2014). However, the molecular mechanisms underlying CSC-associated chemo-resistance and ovarian cancer relapse are not understood fully. Therefore, an understanding of the molecular characteristics of CSCs in ovarian cancer is of critical importance for therapy of patients with ovarian cancer.

The Wnt/β-catenin pathway is involved in various cellular responses, such as stem cell self-renewal and organogenesis (Sweeney et al., 2020; Van Camp et al., 2014). An increasing body of evidence suggests a pivotal role of the Wnt/β-catenin pathway in tumorigenesis and in the development of CSC-like populations (Holland et al., 2013). In the absence of Wnt ligands (in the canonical Wnt/β-catenin pathway), β-catenin gets phosphorylated by the destruction complex, comprising the scaffold protein Axin, APC, glycogen synthase kinase-3β (GSK3β), and casein kinase (Anastas and Moon, 2013; Nusse and Clevers, 2017). The Wnt ligand-induced activation of the Frizzled-LRP5/6 receptor complex abrogates proteasomal degradation of β-catenin, which is induced by GSK3β-dependent phosphorylation of β-catenin at Ser33/37/Thr41 and subsequent ubiquitination, thereby resulting in stabilization of β-catenin (Clevers, 2006). GSK3β can be phosphorylated at Ser9 by various kinases including protein kinase A, protein kinase B (PKB or AKT), p90 ribosomal S6 kinase, and p70 ribosomal S6 kinase. The phosphorylation of GSK3β at S9 leads to its inactivation by proteasomal degradation and subsequent stabilization of β-catenin protein (Cervello et al., 2017). The stabilized β-catenin translocates to the nucleus to form a transcriptional complex with T cell factor (TCF) or lymphoid enhancer factor (LEF) to activate expression of downstream target genes (Clevers, 2006). However, the molecular mechanisms involved in the regulation of the Wnt/β-catenin pathway in CSCs are still elusive.

The tribbles gene family encodes an evolutionarily conserved protein family that influences proliferation, motility, metabolism, and oncogenic transformation (Eyers et al., 2017). Three members of the tribbles family encode serine-threonine kinases; however, they lack the active sites required for kinase activity. Tribbles do not have a catalytic function, but play vital roles in various cellular responses as scaffolds or adaptors to promote the degradation of target proteins and to regulate signaling pathways (Sakai et al., 2016; Salome et al., 2015). Tribbles homolog 2 (TRIB2) promotes or suppresses tumorigenesis in various cancer cells depending on their genetic and epigenetic backgrounds (Eyers et al., 2017; Friedman, 2015; Link, 2015; Yokoyama and Nakamura, 2011). TRIB2 has been implicated as a regulator of proteasomal degradation (Salome et al., 2015). For instance, TRIB2 functions downstream of Wnt/β-catenin signaling to regulate YAP and C/EBPα function and promotes the stability of YAP protein by inhibiting β-TrCP-mediated proteasomal degradation of YAP (Wang et al., 2013). Whereas, TRIB2 has been reported to reduce protein stability of β-catenin and TCF4 and inhibit Wnt activity by acting as a scaffold with its associated E3 ligases, β-TrCP, COP1, and Smurf1 (Xu et al., 2014). Moreover, TRIB2 stimulated C/EBPα degradation mediated by COP1 and TRIM21 E3 ligases (Grandinetti et al., 2011; Keeshan et al., 2006). In addition to its role in proteasomal degradation of signaling molecules, TRIB2 overexpression activated AKT and conferred resistance to anti-cancer therapy via AKT activation and suppression of FOXO3a and p53 (Hill et al., 2017). However, the roles of TRIB2 in the regulation of stemness-like characteristics of CSCs and the Wnt-β-catenin signaling are unclear.

In this study, we investigated the role of TRIB2 in the regulation of CSC-like phenotypes, including the expression of stemness-associated genes, spheroid formation in 3D suspension culture, increased proliferation and migration, drug resistance, and tumorigenic potential. In addition, we showed that TRIB2 promotes the acquisition of CSC-like properties by enhancing β-catenin stability via modulating the AKT-GSK3β-dependent signaling pathway in ovarian cancer.

MATERIALS AND METHODS

Cell culture and analysis of the spheroid-forming ability

A2780, a human ovarian cancer cell line, was purchased from the American Type Culture Collection (USA). A2780 cells were cultured in RPMI-1640 medium supplemented with 10% fetal bovine serum (FBS) at 37°C in 5% CO_2. A2780 ovarian cancer spheroids were isolated from A2780 cells as previously described (Choi et al., 2016). In brief, A2780 cells were seeded at Ultra-Low Attachment 6-well culture plates (Corning, USA) with a density of 2×10^3 cells/10 cm^2, followed by culturing in a serum-free Neuro Basal Medium supplemented with B-27 Supplement, 10 ng/ml basic fibroblast growth factor, 20 ng/ml epidermal growth factor, 2.5 μg/ml amphotericin B, 100 U/ml penicillin, and 100 μg/ml streptomycin. To measure the spheroid-forming ability, the cells were seeded in 24-well Corning ultra-low-attachment plates (Corning) at a concentration of 1,000 cells/ml and cultured in spheroid culture media. After the suspension cells were cultured for 20 days, the number of spheres was counted using a microscope.

Flow cytometry analysis and cell sorting

ALDH (aldehyde dehydrogenase) activity was measured using an Aldefluor assay kit (STEMCELL Technologies, Canada) as recommended by the manufacturer. The fluorescence intensity of the stained cells was analyzed using a flow cytometer (CANTO II; BD Biosciences, USA). ALDH activity of the sample was determined from the fluorescence intensity beyond the threshold defined by the reaction with 4-diethylaminobenzaldehyde. The ALDH-positive and -negative populations were sorted by FACS Aria/Aria III (BD Bioscience), and the ALDH activity of each population was confirmed by using flow cytometry analysis.

Proliferation assay and drug resistance assay

Cell proliferation was determined using a colorimetric 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assay as previously described (Choi et al., 2016). A2780 cells were seeded into a 24-well culture plate at a

density of 1 × 10^4 cells/well under various experimental conditions, respectively. MTT assay was used to determine relative cell growth rate every 24 h for cell growth curves. To measure the drug resistant capacity of A2780 cells to paclitaxel and cisplatin, A2780 cells were plated onto a 96-well cell culture plate in 100 μl RPMI 1640 medium with 2% FBS. After the cells exposed to cisplatin and paclitaxel for 24 h, the viability of cells was determined by MTT assay.

Cell migration assay

Cell migration was measured using a disposable 96 well chemotactic chamber (Neuro Probe, USA). A2780 cells were harvested and suspended in RPMI medium. Membrane filters (8 μm pore size) of the 96 well chemotactic chamber were pre-coated with 20 μg/ml rat-tail collagen (Thermo Fisher Scientific, USA) for 4 h in the clean bench at room temperature. Aliquots (5 × 10^3/50 μl) of A2780 cells were loaded into the upper chambers of chemotactic chamber, and RPMI with 10% FBS was added into the lower chambers. After incubation at 37°C for 12 h, the membrane filter was washed twice with HBSS and fixed with 4% paraformaldehyde. Migrated cells to the lower surface of each filter were stained with Hoechst dye and then measured by microscopic cell count. For the wound scratch assay, A2780 cells from each condition were scratched with a pipette tip to form a wound. Cells were imaged at 0- and 24-h post-scratching and the distance moved by the cells was measured.

Western blotting

Cell lysates for western blot were prepared as previously described (Choi et al., 2016). Briefly, the cell lysate was separated by SDS-PAGE, and then transferred to a nitrocellulose membrane (GE Healthcare, USA). Thereafter, the membrane was blocked with 5% fat-free milk for 30 min and incubated with the corresponding primary antibodies for 1 h. The membrane was incubated with the HRP-conjugated secondary antibody, followed by visualization using an enhanced chemiluminescence reagent (ECL; Amersham Biosciences, USA).

Reverse transcription-polymerase chain reaction (RT-PCR)

Cells were harvested at indicated times, and total RNA was extracted using TRIzol (Sigma-Aldrich, USA). For synthesis of cDNA, 1 μg total RNA from each sample was reverse transcribed into cDNA using an Easy cDNA Synthesis Kit (NanoHelix, Korea) according to the manufacturer's instructions. cDNA (1 μl each) was added into a PCR tube containing HelixAmpTM Ready-2×GO (NanoHelix) and 10 pmol of sense and antisense primers, and performed the PCR with the following thermal cycle: denaturation at 95°C for 30 s, annealing at 52°C-58°C for 30 s depending on the primers used, and extension at 72°C for 30 s. Each PCR reaction was carried out for 25-30 cycles. Primer sequences are listed in Supplementary Table S1. PCR products were analyzed by electrophoresis on a 1% agarose gel.

Short-hairpin RNA-mediated knockdown of genes

The TRIB2-specific lentiviral shRNA construct was obtained from Sigma-Aldrich. Functional sequences in the shRNA vectors are as follows: TRIB2, CCGGCGTCAACATGGAAGAGAACTTCTCGAGAAGTTCTCTTCCATGTTGACGTTTTT. For production of lentivirus, 293FT cells were transfected with target viral plasmid (6.67 μg), VSV-G (5 μg) and Δ8.9 (3.33 μg) using jetPRIME reagent (PolyPlus, USA). Two days after gene transfection, the culture supernatants of 293FT cells containing viral particles were collected. A2780 cells were treated with the culture supernatant for 24 h in the presence of 10 μg/ml Polybrene (Sigma-Aldrich), and followed by puromycin selection.

Tumor xenograft transplantation

All animal studies were conducted in accordance with policies of the National Institutes of Health (NIH) guide for the Care and Use of Laboratory Animals and Institutional Animal Care and Use Committee (IACUC). The animal experimental protocol used in this study received the approval of the Pusan National University IACUC (No. PNU-2018-1816). Mice were bred in pathogen-free animal facility of the Pusan National University under the rigorous monitoring system. Six-week-old female BALB/c-nu/nu mice were obtained from Orient Bio (Korea). In accordance with the guidelines of the Pusan National University pathogen-free animal facility, mice were housed for 1 week acclimation under conditions of 12-h light/dark cycle, controlled temperature (25°C ± 2°C), 50%-60% relative humidity with free access to water and standard rodent chaw. Mice received a subcutaneous injection of A2780 spheroid cells (1 × 10^3 cells/200 μl phosphate-buffered saline [PBS]) infected with a control lentivirus or a sh-TRIB2-bearing lentivirus; all injections were made in the right and left flanks of the mice. After five weeks, mice were euthanized, tumors were weighed, and tumor volumes were measured.

The cancer genome atlas (TCGA) analysis

The supplementary methods for survival analysis from gene expression and clinical outcome data of TCGA ovarian cancer datasets are described in supporting information (Supplementary Materials and Methods).

Statistical analysis

All observed data are presented as mean ± SD. Statistical significance between two groups was analyzed using a two-tailed unpaired Student's *t*-tests. For statistical significance testing of multivariate data sets, one-way or two-way ANOVA with a Scheffé's post hoc test was used.

RESULTS

Increased expression of TRIB2 in the spheroid culture of A2780 ovarian cancer cell line

To explore the role of TRIB isoforms in ovarian CSCs, we measured the expression of TRIB isoforms in the 3D suspension culture of A2780 cells. A2780 cells were dissociated into single cells, cultured with a CSC culture medium on low attachment dishes for 20 days, followed by determination of the mRNA levels of three TRIB isoforms using RT-PCR. The mRNA levels of TRIB2 increased dramatically in a time-dependent manner and correlated significantly with the growth of A2780 spheroids (Figs. 1A and B). The TRIB3 mRNA levels

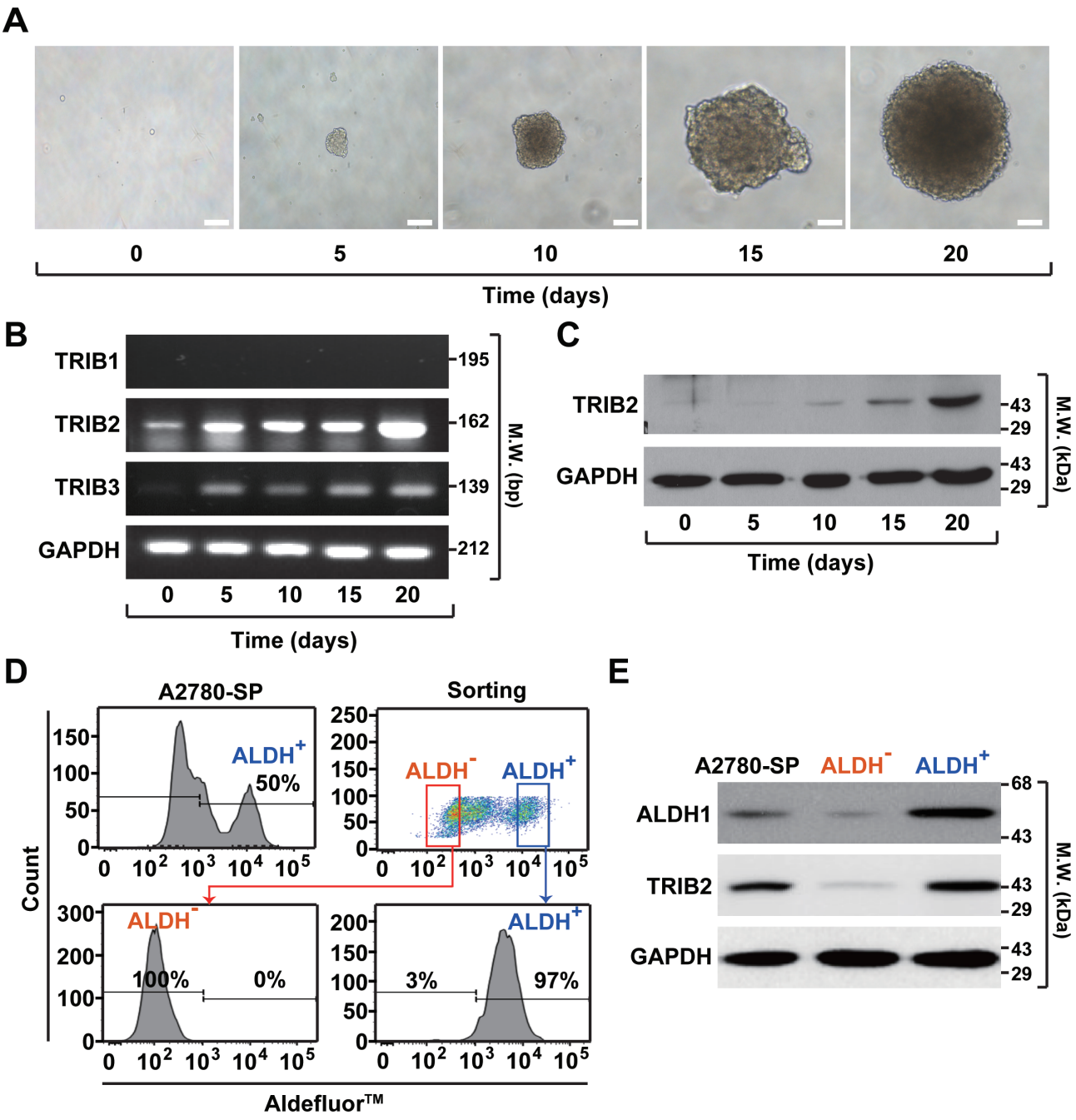

Fig. 1. Increased expression of TRIB2 in the CSC-like population of A2780 ovarian cancer cells. (A) A2780 cells were dissociated into a single cell suspension, cultured in low attachment dish, and photographed at the indicated time points (from day-0 to day-20, magnification, ×100; scale bars = 100 μm). (B) Increased mRNA levels of TRIB2 and TRIB3 in the A2780 spheroids. The mRNA levels of three TRIB isoforms were determined at the indicated time points. M.W., molecular weight. (C) The increased protein level of TRIB2 in the A2780 cell spheroids. The protein levels of TRIB2 and GADPH were determined by western blotting at the indicated time points. (D) Aldefluor activity was measured in the A2780 spheroids by flow cytometry analysis. The ALDH-positive and the ALDH-negative populations indicated as open squares were isolated by cell sorting, and the purity of each population was confirmed by flow cytometry analysis. (E) The protein levels of ALDH1a1, TRIB2, and GAPDH were measured in the A2780 spheroids and their ALDH negative and ALDH positive populations by western blotting.

increased negligibly on day five; however, the increase in TRIB3 expression was considerably lower than that of TRIB2. In contrast to TRIB2 and TRIB3, TRIB1 mRNA in A2780 cells was undetectable by RT-PCR (Fig. 1B). Consistent with the robust induction of TRIB2 mRNA, TRIB2 protein could be observed on day ten by western blotting, and the protein levels

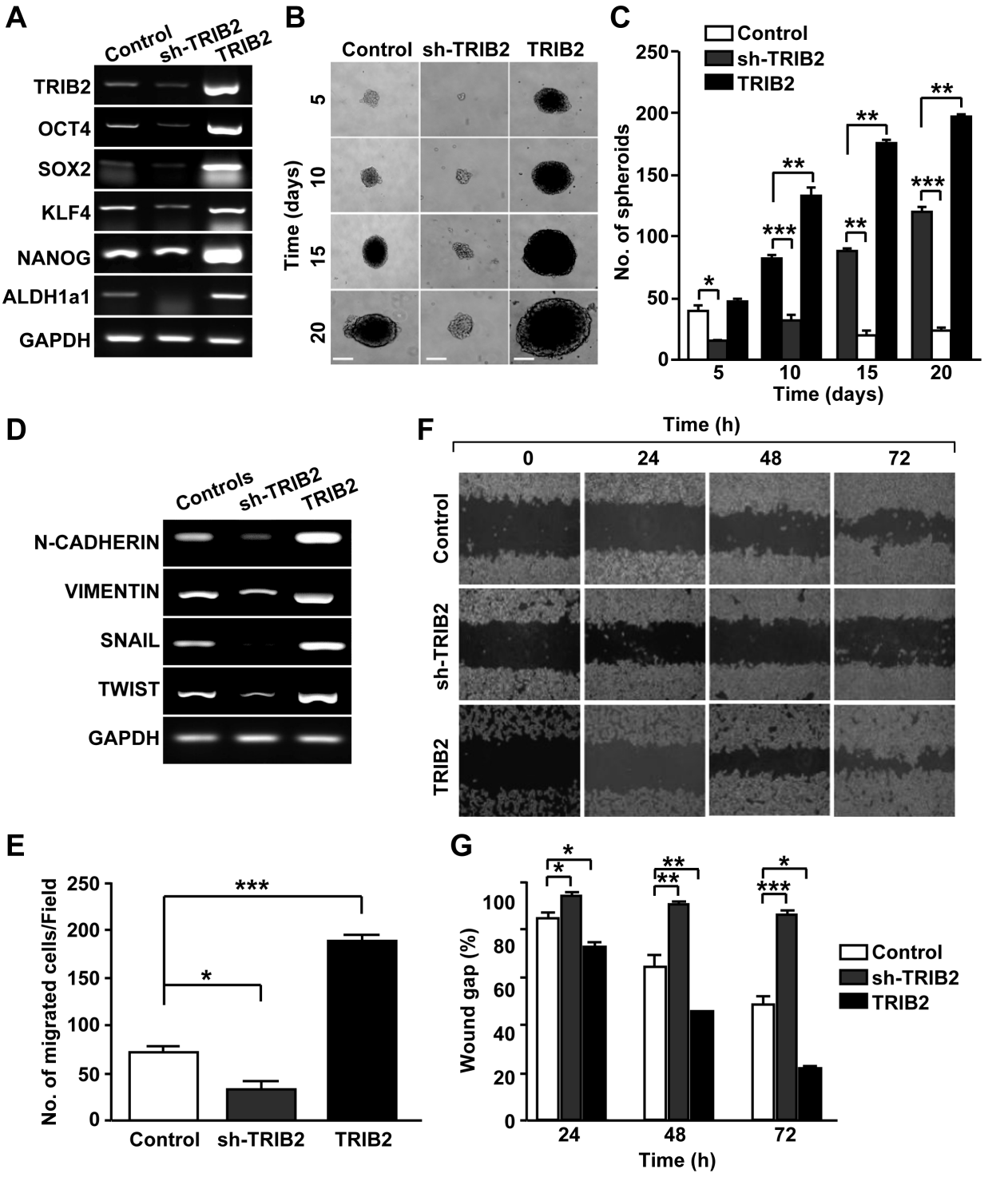

Fig. 2. Effect of TRIB2 on the CSC-like properties of A2780 cells. (A) Effect of TRIB2 silencing or overexpression on the expression of CSC markers. TRIB2 expression was silenced by infection with lentivirus bearing TRIB2 shRNA or overexpressed in A2780 cells and the mRNA levels of TRIB2 and CSC markers (OCT4, SOX2, KLF4, NANOG, ALDH1a1) were determined by RT-PCR analysis. (B) The cells were seeded onto low attachment dishes, and the spheroids photographed at the indicated time points. Scale bars = 100 μm. (C) The number of spheres was counted in the TRIB2-silenced or -overexpressed A2780 cells at the indicated time points. (D) Effects of TRIB2 knockdown or overexpression in the mRNA expression of epithelial-mesenchymal transition-associated genes. (E and F) Effect of TRIB2 silencing or overexpression on the migration of A2780 cells. The migratory abilities of TRIB2-silenced or -overexpressed A2780 cells and their parental A2780 cells were measured by using a Trans-well cell migration assay and wound scratch assay. (G) The wound gaps in the cell migration were measured at the indicated time points. Data are shown as the mean ± SD. $*P < 0.05$; $**P < 0.01$; $***P < 0.001$.

of TRIB2 increased time-dependently in the 3D suspension culture (Fig. 1C), suggesting the upregulation of TRIB2 in A2780-derived spheroids.

We identified the expression of TRIB2 in the CSC-like population of A2780 cells by examining the expression of TRIB2 in a cell population with a high level of aldehyde dehydrogenase activity, a CSC marker (Marcato et al., 2011; Orecchioni and Bertolini, 2016). ALDH activity in A2780 spheroids was assayed using the Aldefluor fluorescent dye by flow cytometry analysis (Fig. 1D). In A2780 spheroids, the ALDH-positive population was measured to approximately 50%. The ALDH-negative and the ALDH-positive populations were isolated by FACS, and the expression of ALDH and TRIB2 was determined by western blotting. The expression of both ALDH1a1 and TRIB2 in the ALDH positive population was higher than that in the ALDH negative population (Fig. 1E). These results suggest that TRIB2 is expressed at considerably high levels in the sphere-forming and ALDH positive CSC population of A2780 cells.

TRIB2 is required for the sphere-forming and migration abilities of A2780 cells

The role of TRIB2 in the regulation of CSC-associated characteristics was evaluated by silencing TRIB2 by infecting with a lentivirus bearing a TRIB2-specific shRNA or overexpression by retroviral infection. Silencing of TRIB2 resulted in decreased expression of not only ALDH1A1 but also of stemness-related genes, such as OCT4, SOX2, KLF4, and NANOG, whereas TRIB2 overexpression led to increased expression of these genes (Fig. 2A). TRIB2 knockdown resulted in reduced size and number of spheroids derived from A2780 cells (Figs. 2B and 2C). In contrast, overexpression of TRIB2 resulted in increased size and number of spheroids, suggesting a critical role of TRIB2 in the proliferation of the sphere-forming CSC population of A2780 cells.

EMT is a crucial feature of CSCs and is implicated in migration and metastasis of these cells (Sato et al., 2016; Singh and Settleman, 2010). The role of TRIB2 in the acquisition of EMT-like properties in epithelial ovarian cancer was explored by analyzing the expression of mesenchymal markers, including N-CADHERIN, VIMENTIN, SNAIL, and TWIST, by RT-PCR. The silencing of TRIB2 reduced, whereas the overexpression of TRIB2 increased, the expression of the mesenchymal markers (Fig. 2D). The role of TRIB2 in the migration ability of CSCs was investigated by measuring the migration ability of A2780 cells in a Trans-well migration assay. Knockdown of TRIB2 resulted in reduced migration of A2780 cells, whereas overexpression of TRIB2 increased it (Fig. 2E). Consistently, the silencing of TRIB2 abrogated the migratory ability of A2780 cells in a wound scratch assay, whereas TRIB2 overexpression accelerated it (Figs. 2F and 2G). These results suggest that TRIB2 plays a pivotal role in the maintenance of the sphere-forming capacity and migratory ability of ovarian CSCs.

TRIB2 is involved in the development of drug resistance in A2780 cells

We explored whether TRIB2 is involved in the development of drug resistance in CSCs by examining the effects of TRIB2 silencing or overexpression on the expression of ABC transporters, which are implicated in drug resistance in cancer. TRIB2 silencing resulted in reduced mRNA levels of several ABC transporters, including ABCB1, ABCG2, and ABCC6, whereas TRIB2 overexpression resulted in increased mRNA levels of these three genes (Fig. 3A). Moreover, TRIB2 silencing resulted in enhanced cell death in response to paclitaxel or cisplatin treatment, whereas TRIB2 overexpression markedly attenuated cell death in response to paclitaxel or

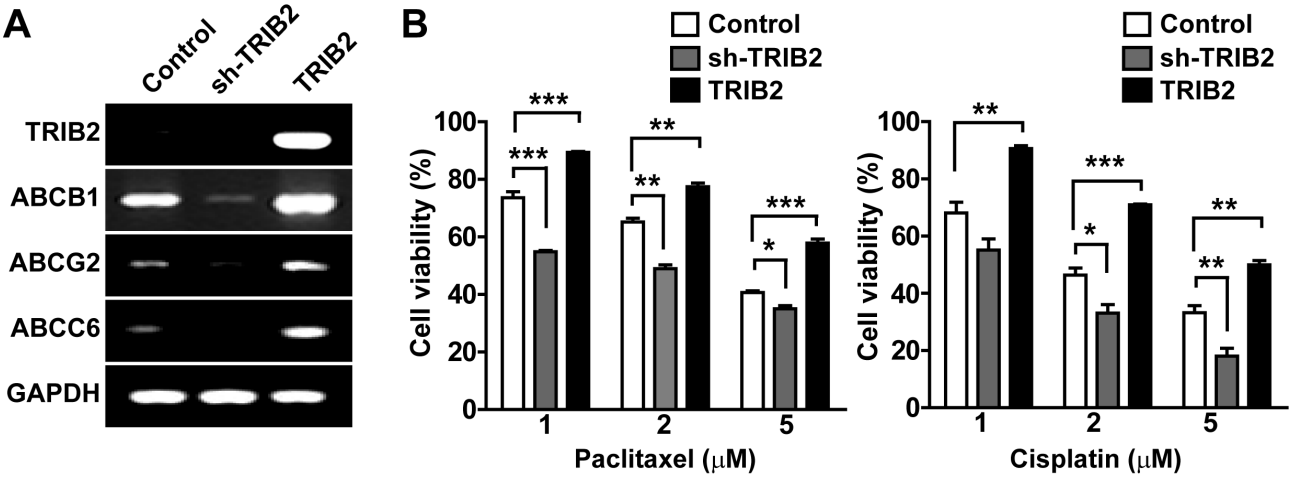

Fig. 3. Role of TRIB2 in the drug resistance of A2780 cells. (A) Effect of TRIB2 silencing or overexpression on the expression of ABC transporters. TRIB2 expression was silenced by infection with lentivirus bearing TRIB2 shRNA or overexpressed in A2780 cells and the mRNA levels of TRIB2, ABC transporters (ABCB1, ABCG2, ABCC6), and GAPDH were determined by RT-PCR analysis. (B) Effect of TRIB2 silencing or overexpression on drug resistance of A2780 cells. The TRIB2-silenced or -overexpressed A2780 cells and the control cells were treated with different doses of paclitaxel (left) and cisplatin (right) for 24 h, followed by cell survival measurement using MTT assay. Data are shown as the mean ± SD. $*P < 0.05$; $**P < 0.01$; $***P < 0.001$.

cisplatin treatment (Fig. 3B). Concordantly, overexpression of TRIB2 increased the activity of the ABCG2 promoter (Supplementary Fig. S1). These results suggest that TRIB2 regulates the transcription of ABC transporter genes, including ABCG2.

TRIB2 is involved in tumor growth *in vivo*

Next, we evaluated the role of TRIB2 in the growth of ovarian CSCs *in vivo*, by examining the effects of TRIB silencing on the tumorigenic potential of A2780 spheroid cells *in vivo*. The A2780 spheroid cells infected with lentiviruses expressing control shRNA or TRIB2 shRNA were transplanted subcutaneously into nude mice, and *in vivo* tumor growth evaluated. Transplantation with TRIB2-silenced A2780 spheroid cells resulted in a significant decrease in the tumor volume *in vivo*, compared to that in the control cells (Fig. 4A). After five weeks, tumors were collected by surgery, and the tumor sizes were found to be significantly smaller (Fig. 4B). These results show an essential role of TRIB2 in tumor growth *in vivo*.

TRIB2 stimulates the acquisition of the CSC-like properties in ovarian cancer through promoting AKT-GSK3β-β-catenin signaling axis

AKT is known to inhibit GSK3β activity by phosphorylating GSK3β at Ser9 (Cross et al., 1995). In addition, TRIB2 induces drug resistance by directly interacting with AKT and promoting the phosphorylation of AKT (Ser473) via its COP1 domain (Hill et al., 2017). For determining whether TRIB2 plays a critical role in AKT-dependent phosphorylation of GSK3β, we examined the effects of TRIB2 overexpression on AKT activation in A2780 cells. Following the TRIB2-dependent decrease of β-catenin phosphorylation levels, TRIB2 overexpression increased the phosphorylation levels of not only AKT but also of GSK3β in A2780 cells (Fig. 5A). Moreover, in 293FT cells, TRIB2 overexpression increased the phosphorylation of AKT and GSK3β, while it decreased the phosphorylation of β-catenin (Fig. 5B).

The involvement of AKT in TRIB2-mediated regulation of the GSK3β-β-catenin signaling pathway was confirmed by examining the effects of the phosphoinositide-3-kinase (PI-3-kinase) inhibitor LY294002 on the phosphorylation levels of the AKT, GSK3β, and β-catenin. Pretreatment with LY294002 abrogated the phosphorylation of AKT and GSK3β augmented by TRIB2 overexpression (Fig. 5C). In contrast, the decrease in β-catenin phosphorylation due to TRIB2 overexpression could be alleviated by LY294002 treatment. Moreover, TRIB2-induced increase in β-catenin protein level was also reduced by LY294002. These results suggest that TRIB2 promotes AKT-dependent phosphorylation and inactivation of GSK3β, leading to decreased phosphorylation and increased stability of β-catenin.

To clarify whether the PI-3-kinase-Akt-GSK3β-β-catenin signaling axis is implicated in the CSC-like properties induced by TRIB2 overexpression, we examined the effects of LY294002 treatment on the spheroid-forming, cell migration, and drug resistance abilities of TRIB2-overexpressed A2780 cells. The TRIB2-stimulated spheroid-forming ability of A2780 cells was significantly attenuated by 10 μM LY294002 treatment (Fig. 5D). Consistently, the TRIB2-stimulated cell proliferation of A2780 cells was also inhibited by 10 μM LY294002 treatment (Fig. 5E). Moreover, the cell migration and doxorubicin-resistant abilities stimulated by TRIB2 overexpression were markedly attenuated by 10 μM LY294002 treatment (Figs.

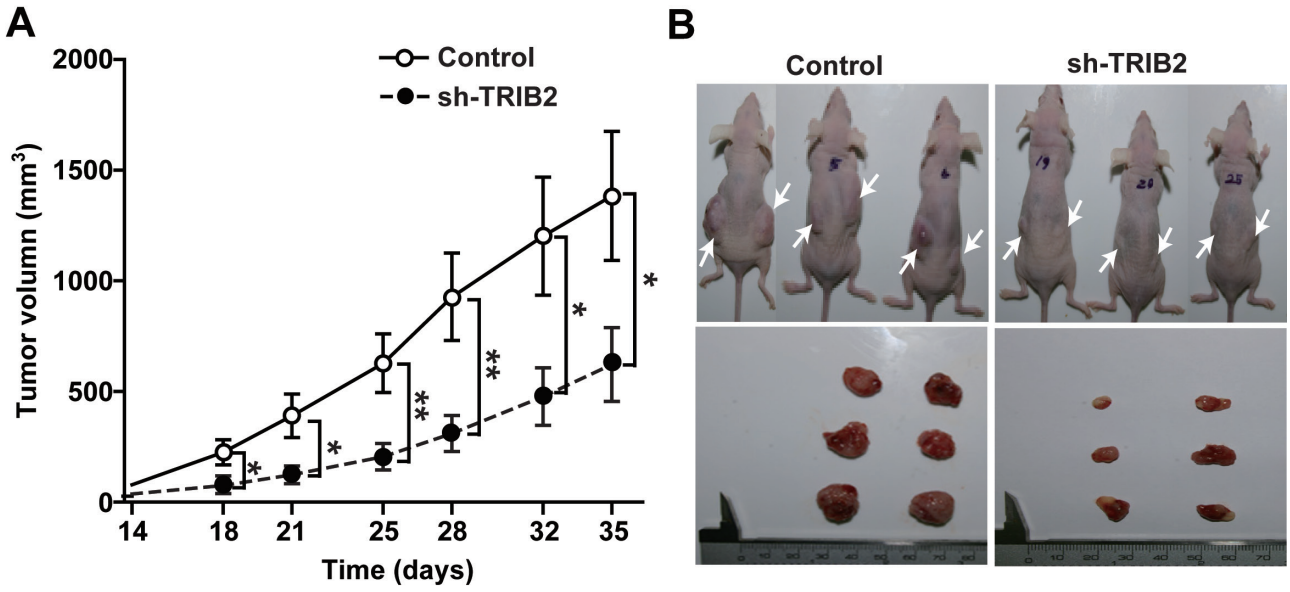

Fig. 4. Effect of TRIB2 knockdown on *in vivo* growth of transplanted A2780 cells xenograft. (A) Inhibitory effect of TRIB2 silencing on tumor growth *in vivo*. The TRIB2-silenced and the control A2780 cells were transplanted into BALB/C nude mice, and the tumor volume measured at the indicated time points. Data are shown as the mean ± SD. $*P < 0.05$; $**P < 0.01$. (B) Representative images of the xenograft tumors on day 35 are shown. The white arrows (upper panel) indicate xenograft tumors, and the tumors isolated on day 35 are photographed (lower panel).

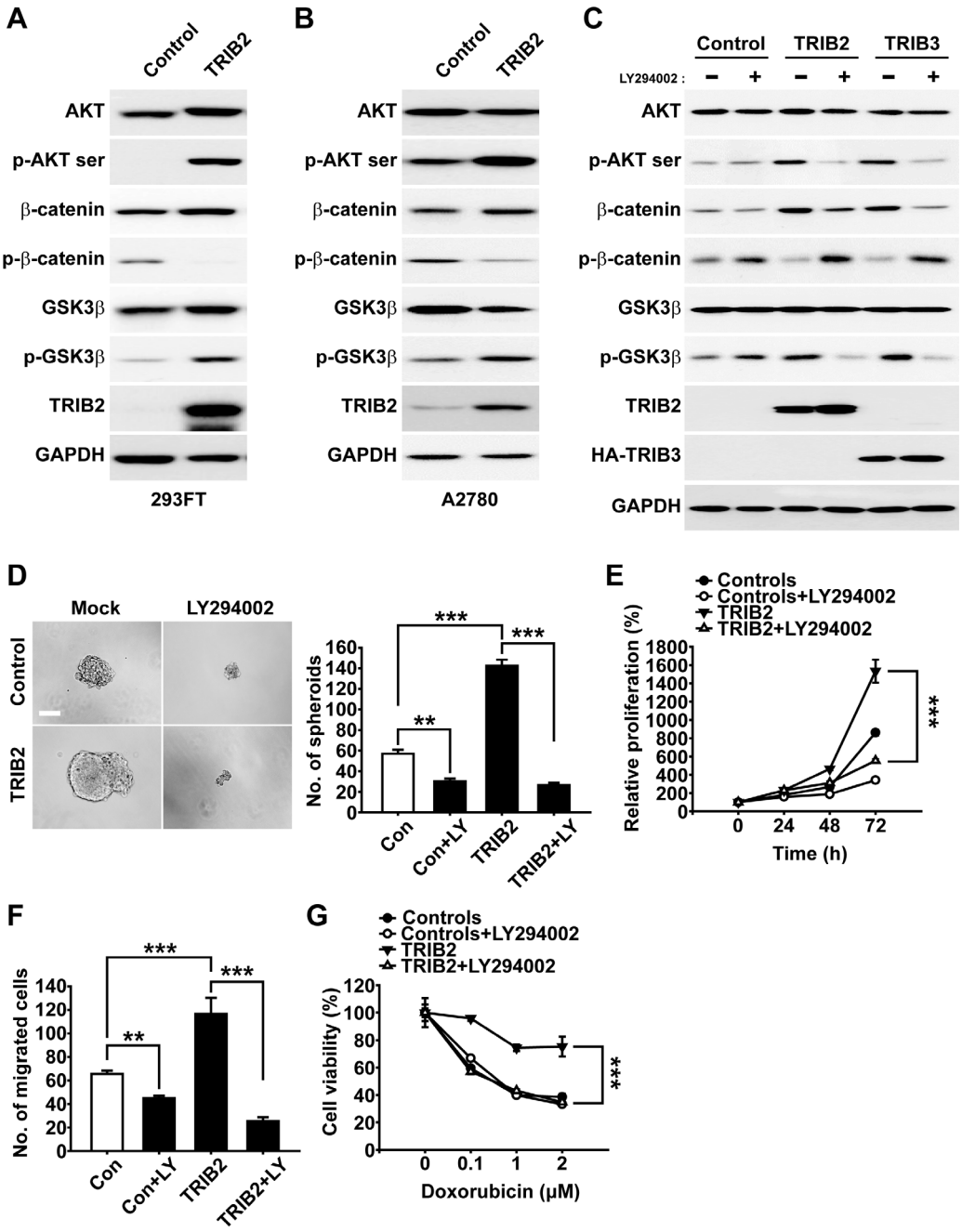

Fig. 5. Role of AKT-GSK3β pathway in the regulation of β-catenin phosphorylation and stability induced by TRIB2. (A and B) Effect of TRIB2 overexpression on the phosphorylation and protein levels of AKT, GSK3β, and β-catenin in 293FT cells (A) and A2780 cells (B). (C) Effect of LY294002 treatment on the phosphorylation and protein levels of AKT, GSK3β, and β-catenin in A2780 cells overexpressing TRIB2 or TRIB3. A2780 cells overexpressing TRIB2 or TRIB3 or control A2780 cells were treated with 10 μM LY294002 for 1 day. The phosphorylation and expression levels of AKT, GSK3β, β-catenin, TRIB2, TRIB3, and GAPDH were determined by western blotting. (D) Effect of LY294002 treatment on TRIB2-stimulated spheroid formation. Control and TRIB2-overexpressed A2780 cells were treated with 10 μM LY294002 for 1 week, and representative images of spheroids are shown (left panel). The number of spheres larger than 50 μm in diameter was counted (right panel, n = 4). (E) Effects of LY294002 treatment on TRIB2-stimulated proliferation. Cell proliferation rate of control and TRIB2-overexpressed A2780 cells were measured by MTT at the indicated times (n = 4). (F) Effect of LY294002 on the TRIB2-stimulated cell migration. The effect of LY294002 on cell migration of TRIB2-overexpressed or control A2780 cells were measured by using a Trans-well cell migration assay (n = 4). (G) Effect of LY294002 on TRIB2-stimulated drug resistance of A2780 cells. TRIB2-overexpressed or control A2780 cells were treated with the increasing doses of doxorubicin in the absence or presence of 10 μM LY294002 cell viability of control and TRIB2-overexpressed A2780 cells were determined by MTT at 24 h (n = 4). Data are shown as the mean ± SD. $^{**}P < 0.01$; $^{***}P < 0.001$.

5F and 5G). These results suggest a pivotal role of the PI-3-kinase-Akt-GSK3β-β-catenin signaling axis in the regulation of CSC-like properties of ovarian cancer cells.

DISCUSSION

An increasing body of evidence suggests that epithelial ovarian CSCs exhibit self-renewing and spheroid-forming abilities, as well as increased expression of stemness markers, including OCT4, SOX2, and NANOG (Liao et al., 2014; Loessner et al., 2010; Peng et al., 2010; Petrik, 2013; Seo et al., 2016). We previously reported considerable expression of the CSC marker ALDH1 and the stemness-associated genes, including OCT4, SOX2, and NANOG in A2780 spheroids (Choi et al., 2016; Seo et al., 2016). Increased expression of TRIB2 has been reported in various human cancer types, including melanoma, lung, liver, and acute leukemias (Lohan and Keeshan, 2013; Yokoyama and Nakamura, 2011). In the present study, we showed upregulation of TRIB2 expression in the spheroid-forming and ALDH positive population of A2780 ovarian cancer cells. Moreover, TRIB2 overexpression led to enhanced spheroid-forming ability *in vitro*. Previously, we reported that TRIB2 regulates the pluripotency of embryonic stem cells and enhances the reprogramming efficiency of somatic cells into induced pluripotent stem cells (Do et al., 2017). TRIB2 interacts directly with OCT4 and increased OCT4-mediated gene transcription. These results strongly suggest that TRIB2 plays a vital role in the regulation of the stemness-associated genes in ovarian CSCs.

The ability of cancer cells to migrate and invade other tissues is a prerequisite for metastasis (Naora and Montell, 2005). Accumulating evidence suggests that CSCs show a high capacity for initiation of epithelial-mesenchymal transition, probably to reduce anoikis and to increase the motility and invasiveness, thus resulting in high tumorigenicity (Cao et al., 2016; Geiger and Peeper, 2009). Using a trans-well migration assay and cell scratch wound assay, we showed that TRIB2 overexpression enhanced cell migration and expression of several EMT-associated genes in A2780. Moreover, TRIB2 silencing in A2780 cells abrogated tumor growth in a xenograft transplantation model. TRIB2 has been identified as a candidate biomarker for melanoma diagnosis and progression (Hill et al., 2017). TRIB2 overexpression accelerated proliferation, cell cycle progression, and blocked cellular senescence in colorectal cancer cells (Ma et al., 2018). TRIB2 and the other TRIB isoforms, i.e., TRIB1 and TRIB3, have been associated with poor prognosis of various cancers (Eyers et al., 2017). Altogether, these reports support the present study that highlights the critical role of TRIB2 in proliferation, migration, EMT, and growth of ovarian CSCs.

The characterization of four molecular subtypes defined ovarian cancer as differentiated, immunoreactive, mesenchymal, and proliferative subtypes according to gene expression signature (Cancer Genome Atlas Research Network, 2011). The mesenchymal subtype overexpressed stemness markers, EMT markers, and Wnt pathway-related molecules (Supplementary Fig. S2). Moreover, the mesenchymal subtype is the poorest survival rate among the four subtypes (Supplementary Fig. S3) (Bamias et al., 2012; Dion et al., 2020; Zhang et al., 2015). The mesenchymal subtype ovarian cancer possesses ovarian CSCs properties. Accordingly, TRIB2 is upregulated in mesenchymal subtype ovarian cancer as well as KLF4, N-cadherin, Vimentin, TWIST, and TCF4. Their expression is significantly correlated with TRIB2 expression in ovarian cancer patients by Pearson's correlation analysis (Supplementary Fig. S4). These results indicated that TRIB2 is closely associated with cancer stemness, EMT, and Wnt pathway in mesenchymal subtype ovarian cancer patients.

Chemotherapy is an important therapeutic strategy for most patients with cancer. Elimination of CSCs, which play a significant role in drug resistance and disease recurrence, is critical to improving cancer treatment outcomes. In the present study, we demonstrated that TRIB2 overexpression caused resistance to cisplatin and paclitaxel and increased the expression of ABC transporters in A2780 cancer cells, suggesting that TRIB2 is a possible target for chemotherapeutics of ovarian cancer. Consistently, the transcriptional activity of the ABCG2 promoter was activated by TRIB2 overexpression. It has been reported that TRIB2-deficient leukemia cells were more resistant to chemotherapy than wild type leukemia cells, with reduced apoptosis and continued propagation, thus suggesting a tumor suppressor role for TRIB2 (Salome et al., 2018). Moreover, TRIB2 knockdown resulted in increased resistance to cisplatin in ovarian cancer cells (Kritsch et al., 2017). Whereas, TRIB2 knockdown inhibited cell growth and reversed drug resistance in human chronic myelogenous leukemia cells (Ma et al., 2018). TRIB2 overexpression induced resistance to cisplatin in small cell lung cancer (Liang et al., 2017), and it caused drug resistance by promoting AKT activation (Hill et al., 2017). Altogether, these results suggest the involvement of TRIB2 in the regulation of the drug-resistant characteristics of CSCs.

In the present study, we demonstrated TRIB2 positively regulated β-catenin protein levels in CSCs. In contrast to the present study, TRIB2 has been reported to negatively regulate Wnt signaling by reducing the protein stability of TCF4 and β-catenin (Xu et al., 2014). Thus, we confirmed by examining the effects of TRIB2 overexpression or silencing on the activity of TCF/LEF-responsive (TOPFlash) promoter. Overexpression of TRIB2 in A2780 cells led to an increase in TOPFlash activity, and knockdown of either TRIB2 or β-catenin expression abrogated TRIB2-induced activation of TOPFlash activity (Supplementary Fig. S5A). Moreover, treatment of A2780 cells with Wnt3a CM, but not control CM, augmented the TOPFlash activity, and silencing of not only β-catenin but also TRIB2 abrogated Wnt3a CM-induced increase in TOPFlash activity (Supplementary Fig. S5B). These results suggest that TRIB2 plays a critical role in Wnt3a-induced expression of β-catenin and subsequent induction of TCF/LEF-dependent gene transcription.

TRIB2 facilitated the nuclear accumulation of its associated E3 ligases and β-catenin, thereby promoting the ubiquitination of β-catenin and TCF4. Recently, it has been reported that TRIB3 overexpression increased the growth of colorectal cancer spheroids by augmenting the transcription of the β-catenin gene without affecting β-catenin stability (Hua et al., 2019). Therefore, we investigated the role of TRIB2 on phosphorylation of β-catenin by examining β-catenin

phosphorylation (Ser33/37/Thr41), which is associated with GSK3β-mediated phosphorylation and proteasomal degradation of β-catenin. TRIB2 silencing resulted in increased phospho-β-catenin level, whereas TRIB2 overexpression decreased it (Supplementary Fig. S6A). Wnt3a CM treatment decreased β-catenin phosphorylation, and TRIB2 silencing attenuated the Wnt3a-induced inhibition of β-catenin phosphorylation (Supplementary Fig. S6B). Overexpression of TRIB2 reduced β-catenin phosphorylation level in the absence or presence of Wnt3a. These results suggest that TRIB2 plays a vital role in the regulation of β-catenin stability by inhibiting the phosphorylation and subsequent degradation of β-catenin.

TRIB3 directly interacted with β-catenin and TCF4, and TRIB3 overexpression increased the recruitment of TCF4 and β-catenin to the promoter region of CSC-associated genes. In addition, TRIB3 activated β-catenin signaling via physical interaction with β-catenin in lung cancer (Hua et al., 2019; Zhang et al., 2019). However, we identified that overexpression of not only TRIB2 but also TRIB3 increased β-catenin stability, suggesting the redundant function of TRIB2 and TRIB3 in the regulation of β-catenin stability. Together with the result that TRIB2 is the primarily expressed isoform of TRIB family members in the A2780 spheroids, the present study suggests for the first time that TRIB2 plays a pivotal role in the regulation of the Wnt/β-catenin signaling pathway by modulating the stability of β-catenin in ovarian CSCs.

The Tribbles act as scaffold proteins to support the modulation of canonical MAPK and AKT modules (Eyers et al., 2017). TRIB3 overexpression suppressed proliferation by reducing the p-AKT level in endometrial cancer cells (Qu et al., 2019). Upregulated TRIB3 binds AKT and inhibits AKT by preventing AKT activation by upstream kinases (Erazo et al., 2016). Loss of TRIB3 is associated with a more aggressive phenotype in a variety of tumors by enhancing the activity of the mTORC2/AKT/FOXO axis (Salazar et al., 2015). However, in the present study, we showed that both TRIB2 and TRIB3 inhibited GSK3β-mediated β-catenin phosphorylation by enhancing AKT-dependent phosphorylation of GSK3β at Ser9. Consistently, TRIB2 overexpression promoted the phosphorylation of AKT at Ser473 via its COP1 domain (Hill et al., 2017). AKT has been known to inhibit GSK3β activity by phosphorylating GSK3β at Ser9 and inducing proteasomal degradation of GSK3β (Cross et al., 1995). We demonstrated that pharmacological inhibition of the PI-3-kinase-Akt pathway abrogated the TRIB2-stimulated proliferation, migration, and drug resistance of ovarian cancer. Altogether, these results support the present study that TRIB2-mediated AKT activation inhibits GSK3β-dependent β-catenin phosphorylation and increases β-catenin protein stability.

Lastly, A recent study reported that the mutation or aberrant expression of β-catenin is improved disease-free survival in ovarian cancer patients (Zyla et al., 2021). Wnt activity was represented to correlate with the grade, EMT, chemo-resistance, and poor prognosis in ovarian cancer patients (Arend et al., 2013; Chau et al., 2013; Jacob et al., 2012; Wang et al., 2006). We found that the expression of TRIB2, β-catenin, and TCF4 in the immunoreactive and mesenchymal subtype ovarian cancer is correlated with poor prognosis (Supplementary Fig. S7). The median survival months of triple-positive patients were 14.62 months, and those of triple-negative patients were 20.34 months. Whereas the median survival months of TRIB2-/beta-catenin+/TCF4+ is 14.42 months, those of TRIB2+/beta-catenin-/TCF4- is 26.1 months (Supplementary Table S2). Overexpression of β-catenin/TCF4 contributes to poor prognosis in TRIB2 low-expressed ovarian cancer patients. Although TRIB2 was overexpressed, the low-expressed beta-catenin/TCF4 ovarian cancer patients showed a good prognosis. These results indicated that TRIB2 could be a novel upstream regulator of beta-catenin/TCF4 (Fig. 5, Supplementary Fig. S7A, Supplementary Table S2). Therefore, the interaction of TRIB2 and the Wnt pathway is substantially associated with ovarian cancer survival rate.

In summary, the present study demonstrates for the first time that TRIB2 plays a pivotal role in the regulation of proliferation, migration, drug resistance, and tumorigenic potentials of ovarian CSCs by increasing β-catenin protein stability. TRIB2 plays a crucial role in the regulation of the Wnt/β-catenin signaling axis via modulating the AKT/GSK3β/β-catenin pathway as a scaffolding protein. The present study suggests that TRIB2 may be a useful target for treating ovarian cancer patients.

Note: Supplementary information is available on the Molecules and Cells website (www.molcells.org).

ACKNOWLEDGMENTS

Support for this research was by the MRC programs (NRF-2015R1A5A2009656) of the National Research Foundation of Korea funded by the Ministry of Education, Science and Technology (NRF-2020R1I1A3073064; NRF-2020R1A2C2011880) and the 2019 Post-Doc Development program of Pusan National University.

AUTHOR CONTRIBUTIONS

D.K.K. designed research, performed experiments, analyzed data, and wrote the manuscript. Y.N.K., Y.E.K., S.Y.L, M.J.S., E.K.D., K.U.C., S.C.K., K.H.K., D.S.S., and P.S. performed experiments and analyzed data. J.H.K. supervised specific experiments and contributed to research designs and manuscript editing.

CONFLICT OF INTEREST

The authors have no potential conflicts of interest to disclose.

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