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TRAF4 promotes endometrial cancer cell growth and migration by activation of PI3K/AKT/Oct4 signaling

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ABSTRACT

Objective: Endometrial cancer (EC) is ranked as the most common gynecologic malignancy of the female genital tract and the fourth most common neoplasia in women. Accumulated evidences reveal that TRAF4 plays a critical role in the progress of various cancers, but its functions in EC remains unclear. This study aimed to explore the role and mechanism of TRAF4 in EC progress.

Methods: TRAF4 expression in EC tissues were assessed by qRT-PCR, IHC and western blot. TRAF4 expression in Ishikawa and primary EC cells was inhibited and overexpressed by transient transfections. Thereafter, cell proliferation was identified by combination of clone formation assay and Ki67 staining assay. Cell viability, apoptosis and migration were respectively measured by MTT assay, flow cytometry assay and transwell migration assay. Furthermore, qRT-PCR and western blot analysis were mainly performed to assess the expression levels of apoptosis-related proteins and PI3K/AKT/Oct4 pathway proteins.

Results: TRAF4 was up-regulated in EC tissues. Knockdown of TRAF4 induced cell apoptosis and inhibited cell proliferation and migration. However, TRAF4 overexpression increased cell proliferation and migration. Furthermore, we found TRAF4 down-regulation repressed the activation of PI3K/AKT signaling pathway in Ishikawa and primary EC cells. We also found that Oct4 was a downstream factor of PI3K/AKT pathway and was positively regulated by TRAF4. TRAF4 might increase cell viability through PI3K/AKT/Oct4 pathway.

Conclusion: The present study demonstrated that TRAF4 might exert an oncogenic role in EC progress via its regulation of PI3K/AKT/Oct4 pathway.

1. Introduction

Endometrial carcinoma (EC), one of the most common gynaecological malignancys of the female reproductive system throughout the world, is diagnosed most frequently in women between 45 and 65 years old and its incidence has increased in recent years (MM et al., 2016; Morice et al., 2016). EC is a heterogeneous disease characterized by dysregulation of cell proliferation and metastasis (Ihira et al., 2017). It could be initiated and promoted by molecular pathways alterations, such as proto-oncogene activation, tumor suppressor gene inactivation, aberrant DNA methylation, and noncoding microRNA (miRNA) dysregulation (Dong et al., 2013). However, the genetic and epigenetic basis of EC is not yet fully understood.

Tumor necrosis factor receptor-associated factors (TRAFs) belong to a family of adapter proteins that are involved in tumor necrosis factor receptor superfamily signaling and share a common structural domain at their C-terminus (Bradley and Pober, 2001; Lu et al., 2003). TRAFs have been identified to regulate cell life and death through directly or indirectly influence the function of many signaling molecules (Bradley and Pober, 2001). TRAF4, an atypical member of the TRAF protein family, was initially identified differential screening of human metastatic lymph nodes in breast carcinoma (Kedinger and Rio, 2007). Previous studies suggested that TRAF4 was amplified and overexpressed and was involved in the initiation and progression of primary breast cancers and metastases (Kornegoor et al., 2012; Régnier et al., 1995). Emerging evidence indicated that TRAF4 was highly expressed in various human malignancies, including breast cancer, lung cancer, colon adenocarcinomas, melanomas, neurogenic tumors, and lymphomas (Camilleribroët et al., 2007; Yao et al., 2014). Besides, TRAF4 has been identified as an oncogene and plays a critical role in cell proliferation, apoptosis, migration and invasion in breast cancer and other kinds of cancer (Wang et al., 2013; Zhang et al., 2013a; Zhang et al., 2014b). However, few studies have focused on the function of TRAF4 in EC, and the mechanism regarding TRAF4's role in tumorigenesis remains unclear.

The present study aimed to assess the expression of TRAF4 in EC

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and explored the role and the underlying mechanism of TRAF4 in EC cell growth and metastasis. We found that the expression of TRAF4 was up-regulated in EC in vitro. Additionally, TRAF4 inhibition repressed cell growth and migration in EC cells, while TRAF4 overexpression exerted an opposite effect. Moreover, we found underlying mechanism of its regulation may be through regulation of PI3K/AKT/Oct4 pathway. This data may provide new insight into the function of TRAF4 as well as its regulatory mechanisms in the pathogenesis of EC.

2. Materials and methods

2.1. The collection of paired tumor and non-tumor tissues and cell culture

Twenty pairs of EC and their corresponding adjacent non-tumor endometrial tissues used in this study were obtained from patients who underwent surgery at Jining No.1 People's Hospital from Feb 2011 to May 2015. All female patients aged from 30 to 65 years, and the average age was 48 years. The study protocol was approved by the ethics committee of Jining No.1 People's Hospital. All patients had no preoperative neoadjuvant treatment. Tissue specimens were immediately frozen in liquid nitrogen after surgery and preserved at −80 °C for subsequent testing. The specimen collection procedure was performed with written informed patient consent and the approval of the Medical Ethics Committee.

The human EC cell lines, Ishikawa, was obtained from Japanese Cancer Research Resources Bank (JCRB, Osaka, Japan) and were maintained in Dulbecco's modified Eagle Medium F12 (DMEM/F12) (Sigma-Aldrich, St. Louis, MO, USA) supplemented with 10% fetal bovine serum (FBS, Hyclone, Logan, UT, USA), and 1% penicillin-streptomycin solution (Gibco, Life Technologies, Grand Island, NY) at 37 °C in an humidified atmosphere containing 5% CO_2.

Primary EC cells culture was operated according to previous study (Genç et al., 2007). Endometrium tissue was minced and dissociated with collagenase B (1 mg/ml) and DNAase (2 mg/ml) (Roche, Basel, Switzerland) at 37 °C for 30 min. Then cells were separated and centrifugated, washed twice with DMEM/F12 (Sigma-Aldrich) containing 1% penicillin-streptomycin solution (Gibco) and 10% FBS (Hyclone), and transferred into plastic culture flasks (25 cm^2, TPP, St. Louis, MO, USA). Endometrial cells were plated in DMEM-F12 medium and maintained at 37 °C in a humidified chamber containing 5% CO_2, and allowed to replicate until confluent in monolayer. Culture medium was replaced twice a week.

2.2. Immunohistochemical (IHC) analysis

Specimens were confirmed by hematoxylin and eosin-stained sections. Formalin-fixed, paraffin-embedded sections (4 μm) were deparaffinized in xylene, rehydrated in graded alcohol, and rinsed in phosphate-buffered saline (PBS). Endogenous peroxidase activity was blocked with 3% hydrogen peroxide in methanol for 20 min. Epitope retrieval was performed in citrate buffer for 5 min at 100 °C. Slides were incubated with rabbit monoclonal primary antibodies (Cell Signaling Technology, Beverly, MA, USA) diluted to 1:30 at 4 °C overnight. After washing three times with fresh PBS, the sections were subsequently incubated with secondary antibody (Cell Signaling Technology) at room temperature (37 °C) for 30 min. For visualization of the reaction, the diaminobenzidine-tetrahydrochloride was stained, then counterstained with hematoxylin, dehydrated and cover slipped. Two observers without knowledge of the studies' data performed evaluation of the staining.

2.3. Transfection of TRAF4 expressing vector and shRNA

For the analysis of TRAF4 functions, the full-length TRAF4 sequences and short-hairpin RNA (shRNA) directed against TRAF4 were constructed in pcDNA3.1 (Invitrogen), and were referred as to pc-TRAF4 and sh-TRAF4. The Lipofectamine 3000 reagent (Invitrogen) was used for the cell transfection according to the manufacturer's instructions. A non-targeting sequence of plasmid and an empty plasmid were respectively used as negative controls (NCs) of pc-TRAF4 and sh-TRAF4, and referred as to pcDNA3.1 and sh-NC. The stably transfected cells were selected by the culture medium containing 0.5 mg/ml G418 (Sigma-Aldrich). After approximately 4 weeks, G418-resistant cell clones were established.

2.4. siRNA transfection

Oct4 siRNA and control siRNA (si-NC) were purchased from Cell Signaling Technology (Beverly, MA, USA). Cells infected with pc-TRAF4 were incubated with a mixture of siRNA and Lipofectamine 2000 reagent (Invitrogen) in 100 ml of serum-free OPTI-MEM (Invitrogen) according to the manufacturer's instructions.

2.5. Quantitative real time RT-PCR analysis

Total RNA was extracted using TRIzol reagent (Invitrogen), and was reverse transcribed into cDNA using a Reverse Transcription Kit (Takara, Dalian, China) according to the manufacturer's instructions. The expression of TRAF4 was conducted by qRT-PCR using the SYBR Green Master Mix (Takara) according to manufacturer's instruction. The mRNA of GAPDH level was used as an internal control and relative expression changes were calculated using the $2^{-\Delta\Delta Ct}$ method.

2.6. Western blot

The proteins used for western blot were extracted from tissues and cells using RIPA lysis buffer (Beyotime Biotechnology, Shanghai, China) supplemented with protease inhibitors (Roche). Equal amount of denatured protein (30 μg) was loaded and separated by sodium dodecyl sulfate-polyacrylamide gel (SDS-PAGE), and then transferred to polyvinylidene fluoride membranes (Millipore, Bedford, MA, USA). Membranes were blocked with 5% BSA in TBST at 37 °C for 60 min and incubated respectively with the primary antibodies at 4 °C overnight. The primary antibodies: anti-TRAF4 (#18527), anti-Bid (#8762), anti-Bax (#5023), anti-Cleaved caspase 3 (#9662), anti-GAPDH (#2118), anti-p-AKT (#4060), anti-AKT (#4685), anti-p-PI3K (#4228), anti-PI3K (#4249), anti-Oct4 (#2750) were purchased from Cell Signaling Technology and were prepared in 5% BSA at a dilution of 1:1000. After washing with TBST buffer for three times, the membranes were incubated with HRP-conjugated secondary antibodies (Sigma-Aldrich) at a 1:5000 dilution for 2 h at room temperature. ECL reagent (Beyotime Biotechnology) was used for detection. The bolts were visualized by using Image Lab™ Software (Bio-Rad, Hercules, CA, USA).

2.7. MTT assay

The cell viability was evaluated by a 3-(4, 5-dimethylthiazol-2-yl)-2, 5-diphenyltetrazolium bromide (MTT) colorimetric assay (Sigma-Aldrich) according to the manufacturer's instructions. Cells infected with sh-TRAF4 and pc-TRAF4 were seeded into 96-well plates. After 1, 2, 3 and 4 day, cell were washed twice with PBS and added with MTT (5 mg/ml) in the dark and incubated at 37 °C for another 3 h. Then, 100 μl DMSO was added to dissolve the formazan crystals. The optical density of each well was measured at a wavelength of 570 nm on a microplate reader (Bio-Rad Laboratories, Orlando, FL, USA).

2.8. Colony formation assay

For the colony formation assay, 200 cells were seeded into 6-well plate culture dishes and incubated at 37 °C for 2 weeks. After rinsing twice with PBS, the colonies were fixed with ice methanol and stained with 10% Giemsa (Sigma-Aldrich), and the number of visible colonies

was counted.

2.9. Ki67 positive cell detection by flow cytometry assay

Cells were fixed with 70% ethanol for 30 min, washed, and incubated with 1% BSA for 20 min. Then cells were incubated with 1:50-diluted anti-Ki67-antibodies (#11882, Cell Signaling Technology) for 60 min at 4 °C and with 1:1000-diluted Alexa Fluor 488–conjugated goat anti-Rabbit IgG (Invitrogen) for another 30 min. Flow cytometric analysis was performed with a FACScan (Beckman Coulter, Fullerton, CA, USA). The data were analyzed by using FlowJo software (Tree Star Inc., Ashland, OR).

2.10. Apoptosis assay

For apoptosis assay, cells were stained with propidium iodide (PI) and FITC-conjugated annexin V using an Annexin V-FITC/PI Apoptosis Detection Kit (Beyotime Biotechnology). Briefly, transfected cells were harvested and washed with cold PBS and resuspended with 1 × binding buffer at a concentration of 1×10^6 cells/ml. Then, 5 μl PI and 5 μl Annexin V-FITC were added into cell buffer solution and incubated for 15 min at room temperature in the dark. Treated cells were washed twice with cold PBS and resuspended in buffer. Flow cytometry analysis was done by using a FACS can (Beckman Coulter). The data were analyzed by using FlowJo software (Tree Star Inc.).

2.11. Migration assay

Transwell migration assays were performed using 8.0-μm pore inserts (BD Biosciences, San Jose, CA, USA). A total of 2.5×10^4 cells were suspended in 200 μl serum-free medium and loaded into upper wells; and 600 μl complete medium with 10% FBS was added to the lower chambers as a chemoattractant. Cells were incubated for 48 h at 37 °C, the migrated cells were fixed with 4% paraformaldehyde and stained with 0.1% crystal violet solution (Sigma-Aldrich). The cells above the upper surface that did not migrate through the pores were cleared with a cotton swab. The number of migratory cells was confirmed through counting five random areas of constant size/well. All experiments were completed in triplicate.

2.12. Statistical analysis

All experiments were repeated three times. The results of multiple experiments are presented as the mean ± SD. Statistical analyses were performed using Graphpad 6.0 statistical software (GraphPad Software, San Diego, CA, USA). The *P*-values were calculated using a one-way analysis of variance (ANOVA). A *P*-value of < 0.05 was considered to indicate a statistically significant result.

3. Results

3.1. TRAF4 was up-regulated in human EC tissues

The mRNA expression of TRAF4 was significantly up-regulated in human EC tissues when in comparison with the adjacent normal endometrial tissues of patients ($P < .001$) (Fig. 1A). Then we determined the expression of TRAF4 in tumor tissue and adjacent tissue. Interestingly, we found that TRAF4 was expressed in normal tissues and endometrial cancer: The positive rate of TRAF4 cytoplasm was gradually increased in normal tissues and cancer without statistical significance. However, for positive rate of nucleus, the positive rate of nucleus decreased gradually, and the normal tissue was significantly higher than the cancer tissue (Fig. 1B). We further assessed the protein level of TRAF4 in human EC tissues, and western bolt analysis showed that TRAF4 protein expression was highly expressed in randomly selected 5 paired EC tissues compared with respective adjacent normal tissues (Fig. 1C).

3.2. Expression of TRAF4 after transfection with sh-TRAF4 and pc-TRAF4 in EC cells

To characterize the functional importance of TRAF4 in EC tumorigenesis, we knocked down or overexpressed TRAF4 through infection with sh-TRAF4 or pc-TRAF4 in Ishikawa cells. As expected, the mRNA level of TRAF4 was decreased after treatment with sh-TRAF4 and was increased with pc-TRAF4 ($P < .01$, or $P < .001$) (Fig. 2A). Simultaneously, TRAF4 protein expression results were similarly observed in transfected cells measured by western blot analysis (Fig. 2B).

3.3. Knockdown of TRAF4 inhibited cell growth and migration in EC cells

We further investigate the role of TRAF4 in EC cell growth and migration. As shown in Fig. 3A, knockdown of TRAF4 resulted in a significant increase in the number of apoptotic cells ($P < .01$). Western blot analysis showed that TRAF4 knockdown increased the protein expression of pro-apoptosis related factors, such as Bid, Bax and Cleaved caspase3 (Fig. 3B). Ki67 is a nuclear protein that is special expressed in proliferating cells and may be required for maintaining cell proliferation (Yao et al., 2014). We further analyzed the Ki67 positive cell rate and revealed that TRAF inhibition marked reduced the rate of Ki67 positive cell ($P < .001$) (Fig. 3C). Additionally, we also found that inhibition of TRAF4 could decrease relative migration of EC cells ($P < .05$) (Fig. 3D). Furthermore, we detected the effects of TRAF4 silence on cell viability. Interestingly we found that TRAF4 silence decreased cell viability compared with NC. On the other side, TRAF4 silence significantly increased cell viability in a time-dependent manner which indicated that the suppressing effects were weakened in the delaying of the transfection time (Fig. 3E). Finally, we also determined the effects of TRAF4 silence on cell colony numbers (Fig. 3F). Taken together, knockdown of TRAF4 inhibited EC cell growth.

3.4. Overexpression of TRAF4 promoted cell proliferation and migration in EC cells

We further overexpressed TRAF4 in EC cells through transfection with TRAF4 overexpressing vector, and explored the effect of TRAF4 overexpression on cell proliferation and migration. Similarly, we detected the expression of TRAF4 overexpression on cell growth or development factors. Interestingly, almost to the opposite results were led by TRAF4 overexpression compared with TRAF4 silence. No significant results were observed by TRAF4 on cell apoptosis by TRAF4 overexpression (Fig. 4A). This was also shown in the related apoptotic proteins (Fig. 4B). However, the Ki67 positive cell rate ($P < .05$, Fig. 4C) and migration ($P < .05$) (Fig. 4D) was increased by TRAF4 overexpression. On the other side, TRAF4 overexpression increased cell viability on the third and fourth day ($P < .05$ or $P < .01$) (Fig. 4D) and also presented an increasing trend in a time-dependent manner (Fig. 4E). In the end, we found that colony formation analysis also indicated that cells transfected with pc-TRAF4 vector yielded more colonies than the control vector-transfected cells ($P < .01$) (Fig. 4F). In a word, TRAF4 overexpression promoted EC cell growth and development.

3.5. TRAF4 activated PI3K/AKT signaling pathway

The PI3K/AKT signaling plays a pivotal role in the development of tumor by regulation of cell proliferation, colony formation, cell invasion and metabolism (Brazil et al., 2002; Li et al., 2012; Liu et al., 2009). Thus, we further investigated the role of TRAF4 in regulation of PI3K/AKT signaling cascade. As shown in Fig. 5A, TRAF4 inhibition reduced the protein expression of p-AKT and p-PI3K, with no difference of AKT and PI3K. On the contrary, TRAF4 overexpression activated

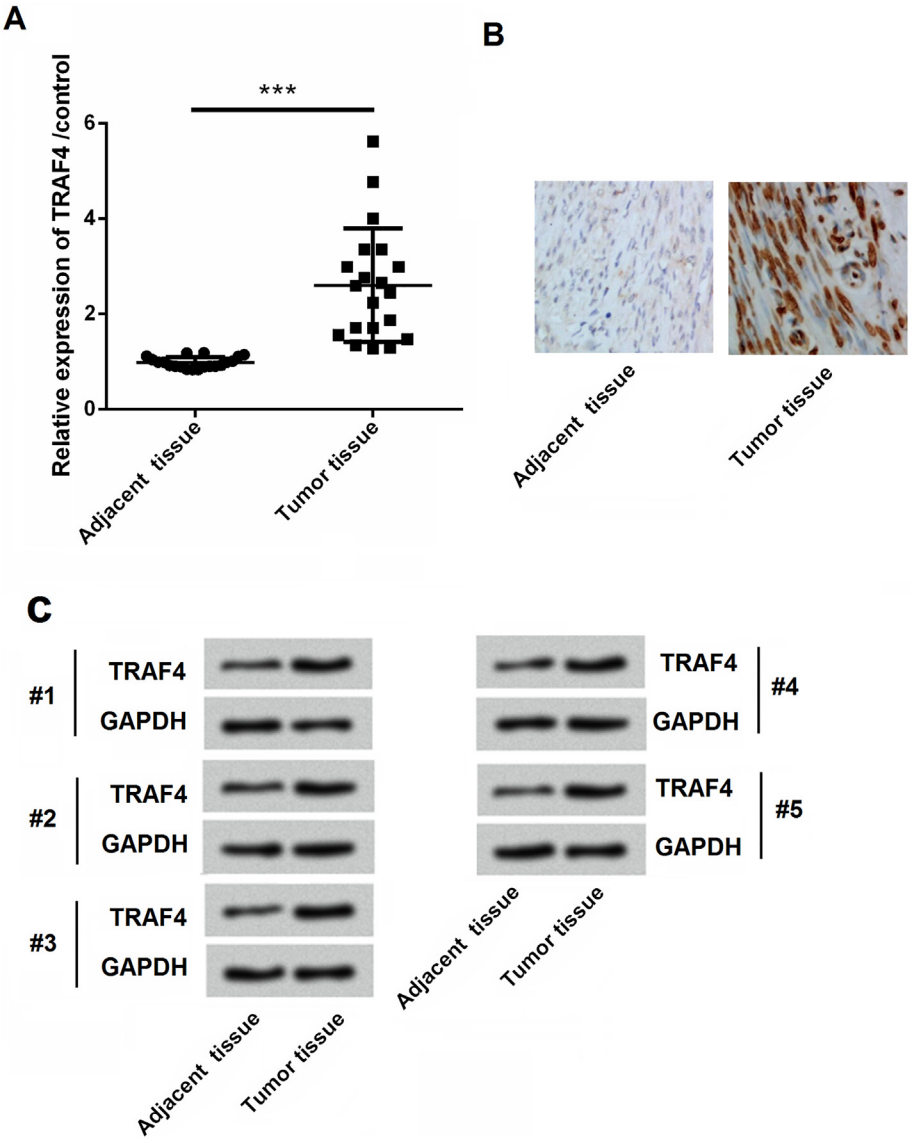

Fig. 1. TRAF4 was up-regulated in human EC tissues. (A) TRAF4 mRNA level was significantly higher in human EC tissues than in adjacent-normal endometrial tissues ($n = 20$). (B) The expression of TRAF4 was detected by IHC. (C) The protein expression of TRAF4 was up-regulated in five human EC tissues in comparison with adjacent-normal endometrial tissues. *** $P < .001$.

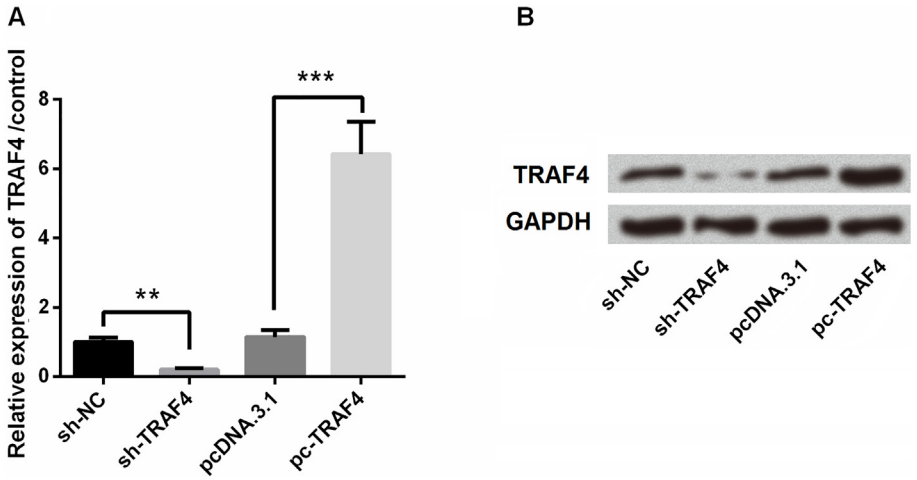

Fig. 2. Expression of TRAF4 after transfection with sh-TRAF4 and pc-TRAF4 in EC cells. (A) Knockdown of TRAF4 by shRNA transfection showed notably expression inhibited and overexpression of TRAF4 by transfection with pc-TRAF4 showed notably expression promoted in mRNA levels ($n = 3$). (B) TRAF4 protein expression was decreased in sh-TRAF infected cells and increased in pc-TRAF4 infected cells. ** $P < .01$, *** $P < .001$.

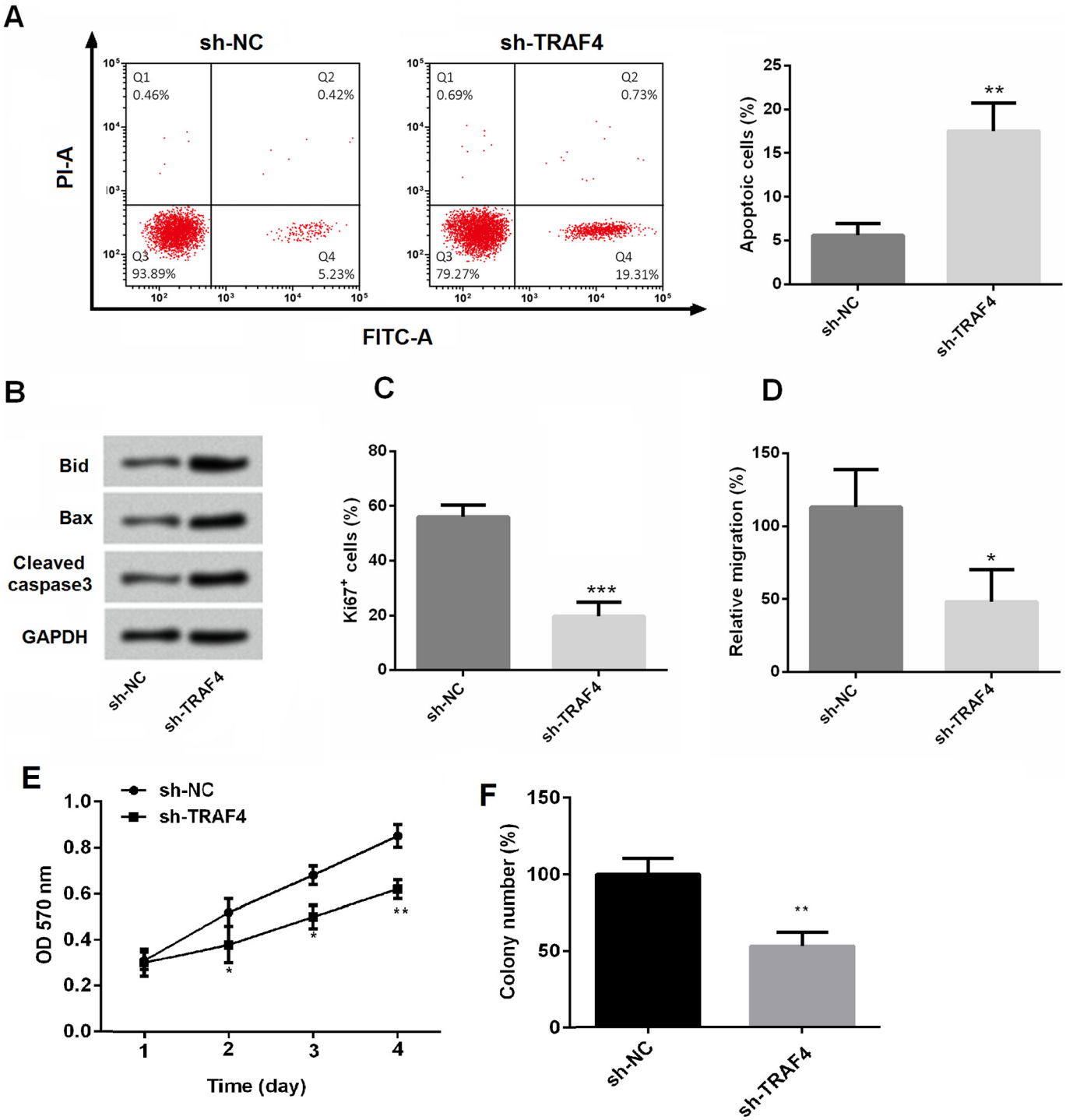

Fig. 3. Knockdown of TRAF4 inhibited cell growth and migration in EC cells. (A) Knockdown of TRAF4 by shRNA transfection showed increased apoptotic rate of EC cells by Annexin V-FITC/PI assay (n = 3). (B) Western blot analysis showed that TRAF4 inhibition elevated the expression of Bid, Bax and Cleaved caspase3. (C) Ki67 staining was performed and the percentage of Ki67 positive ($Ki67^+$) cells was calculated by flow cytometry analysis, and the percentage of $Ki67^+$ cells was declined in sh-TRAF4 infected cells compared with sh-NC (n = 3). (D) TRAF4 inhibition suppressed cell migration in Ishikawa cells (n = 3). (E) TRAF4 silence decreased cell viability and (F) colony cell number. * $P < .05$, ** $P < .01$, *** $P < .001$.

PI3K/AKT signaling pathway, as evidenced by promoting the phosphorylation of AKT and PI3K (Fig. 5B). What's more, we also assessed the effect of TRAF4 on EC tissues. Primary EC cells isolated from random two EC tissues were transfected with sh-TRAF4 and sh-NC. Protein expression of PI3K/AKT signaling related factors results were similarly observed in Ishikawa by western bolt analysis (Fig. 5C). Above all, these results showed that TRAF4 silence inactivated AKT/PI3K signaling pathway while TRAF4 overexpression activated this pathway.

3.6. TRAF4 increased cell viability through regulation of PI3K/AKT/Oct pathway

Previous study has reported that Oct4 is a downstream factor of PI3K/AKT signaling pathway (Teng et al., 2014), thus we further assessed the expression of Oct4 in Ishikawa after TRAF4 was inhibited by sh-TRAF4 transfection. We found that Oct4 expression was reduced when TRAF4 was down-regulated (Fig. 6A). Of contrast, TRAF4

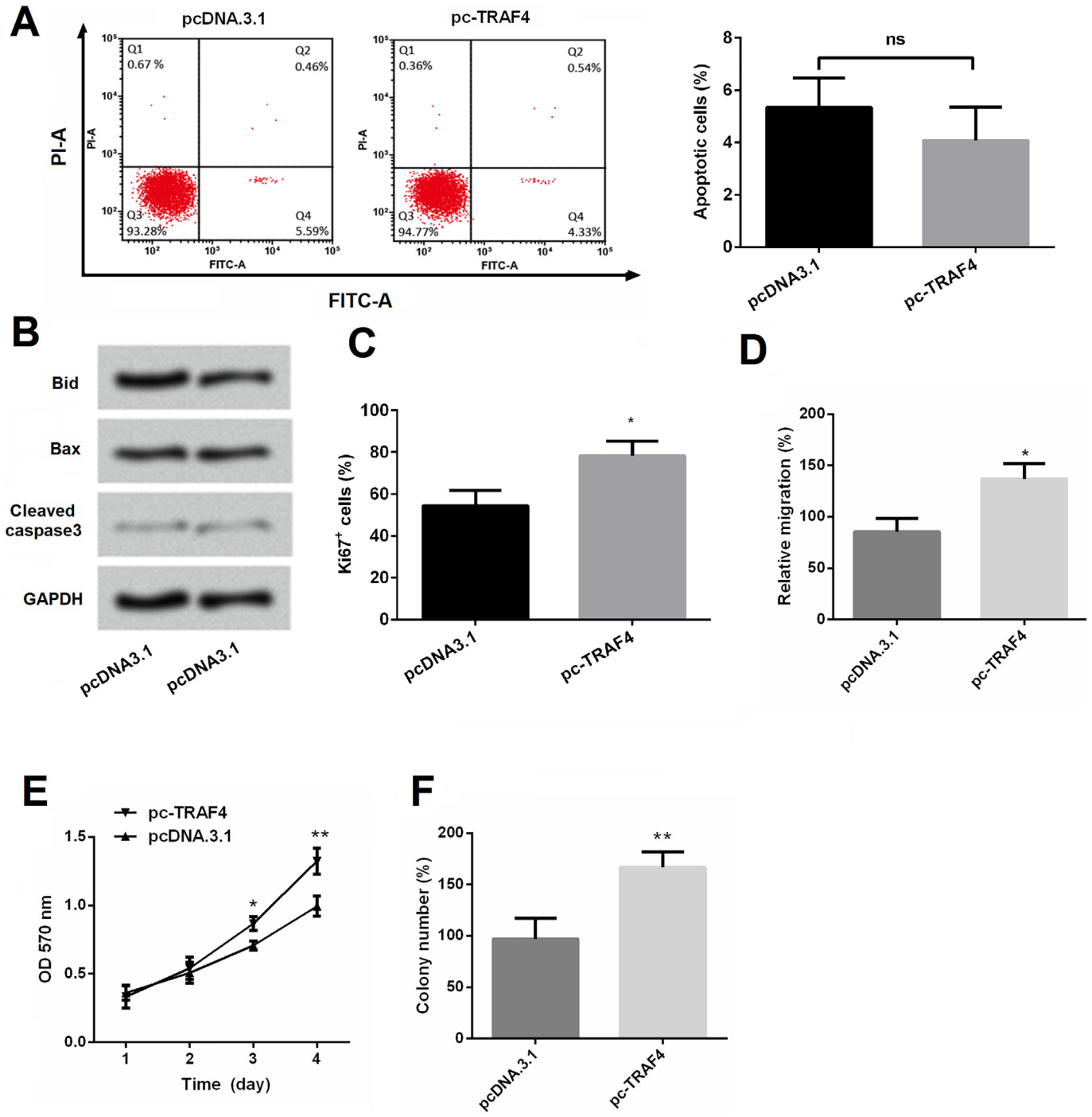

Fig. 4. Overexpression of TRAF4 promoted cell proliferation and migration in EC cells. (A) Overexpression of TRAF4 by pc-RNA transfection showed no difference in apoptotic rate of EC cells by Annexin V-FITC/PI assay (n = 3). (B) Western blot analysis showed that TRAF4 overexpression had no obviously influence on the expression of Bid, Bax and Cleaved caspase3. (C) Ki67 staining was performed and the percentage of Ki67 positive ($Ki67^+$) cells was calculated by flow cytometry analysis, and the percentage of $Ki67^+$ cells was increased in pc-TRAF4 transfected cells compared with pcDNA3.1 (n = 3). (D) TRAF4 overexpression increased cell migration in Ishikawa cells (n = 3). (E) TRAF4 overexpression increased cell viability and (F) colony cell number. * $P < .05$, ** $P < .01$, ns: no significant difference.

overexpression increased the protein level of Oct4. Moreover, we found that LY294002, a specific inhibitor of PI3K/AKT pathway, reduced the expression of p-AKT and p-PI3K, and blocked the promotion of TARF4 overexpression on Oct4 expression (Fig. 6B). In addition, we found that Oct4 expression was positively correlated with the level of TRAF4 in EC tissues (Fig. 6C). MTT assay analysis revealed that TRAF4 overexpression significantly increased cell viability after transfected with pc-TRAF4 vector for 3 day. However, suppression of Oct4 by siRNA reversed the effect of TRAF4 overexpression on cell viability ($P < .05$, or $P < .01$) (Fig. 6D).

4. Discussion

Accumulating evidence indicates that TRAF4 acts a crucial role in the genesis and progress of a range of human malignancies and tumors (Camilleribroët et al., 2007; Gu et al., 2007; Zhang et al., 2013ba). It

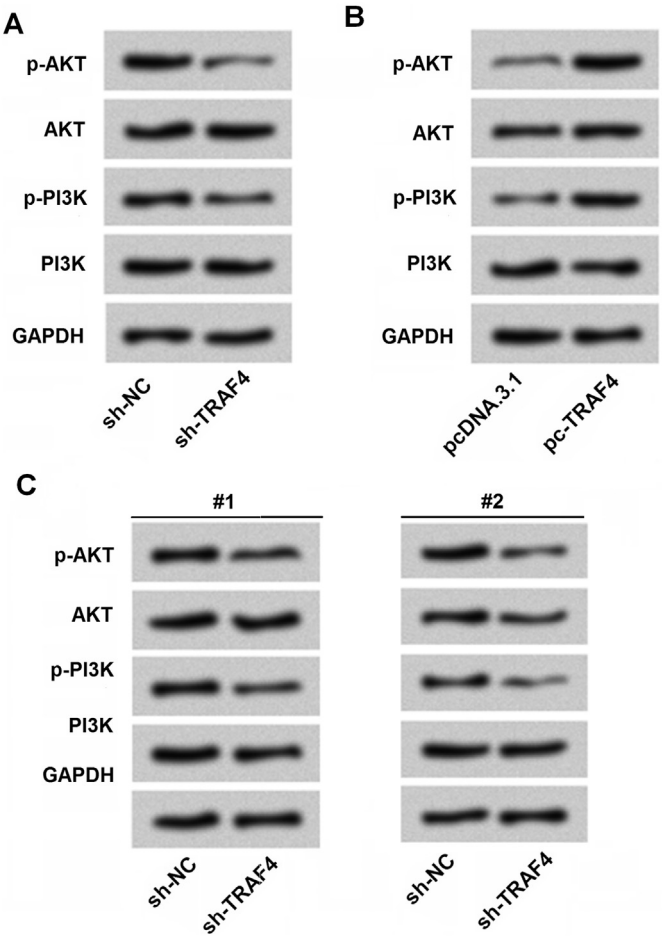

Fig. 5. TRAF4 activated PI3K/AKT signaling pathway. Ishikawa and primary EC cells were transfected with sh-TRAF4, pc-TRAF4 or their corresponding controls, i.e., sh-NC and pcDNA3.1. (A) Western blot analysis showed that TRAF4 knockdown inhibited the activation of PI3K/AKT signaling pathway effectors in Ishikawa. (B) TRAF4 overexpression increased the protein level of p-AKT and p-PI3K in Ishikawa cells by western blot. (C) TRAF4 knockdown inhibited the activation of PI3K/AKT signaling pathway effectors in primary EC cells from two EC tissues by western blot.

has been reported that TRAF4 is overexpressed and functions both as a negative regulator of tight junctions and a promoter of migration in breast cancer. Of interest, TRAF favors cell migration through its ability to interact with phosphoinositides (PIPs) in breast cells (Rousseau et al., 2013). Moreover, TRAF4 accelerates breast cancer metastasis via activation of TGF-β receptor signaling and its expression is closely related with a poor prognosis among breast cancer patients (Zhang et al., 2013a). In particular, TRAF4 is a downstream gene of steroid receptor coactivator 3 (SRC-3) and its expression is positively correlated with SRC-3 expression. And overexpression of TRAF4 diminished cytotoxic stress-induced up-regulation of the tumor suppressor p53 protein and leads to the loss of p53 deubiquitination, thus resulting in cancer cell survivial and growth (Yi et al., 2013). TRAF4 expression is also obvious up-regulated in osteosarcoma tissues and cell lines. Importantly, TRAF4 overexpression dramatically enhances osteosarcoma cell proliferation and invasion partially by activating AKT signaling cascade (Yao et al., 2014). In lung cancer, TRAF4 expression is similar with in breast and osteosarcoma which showing highly expressed, and attenuation of TRAF4 expression by siRNA blunts the malignant phenotype, exerting inhibitory effects on cell proliferation, anchorage-independent growth and tumor development (Li et al., 2013). In agreement with previous studies showing that TRAF4 was highly expressed in human cancer cells (Camilleribroët et al., 2007; Yao et al., 2014), our results showed that TRAF4 expression was significant higher in EC tissues than adjacent normal tissues. And TRAF4 overexpression increased cell viability, colony formation and migration, while TRAF4 down-regulation induced cell apoptosis and reduced cell migration in EC cells.

The PI3K/AKT pathway has been identified to be substantially correlated with essential characteristics for cancer progression and metastasis, such as cell apoptosis, proliferation, migration and invasion (Fresno Vara et al., 2004; Hennessy et al., 2005; Hou et al., 2014; Luo et al., 2003). In EC, the PI3K/AKT pathway has been implicated in the pathogenesis of endometrial cancer and inhibition of the pathway is being vigorously pursued as a potential therapeutic target (Hou et al., 2014; Li et al., 2016; Slomovitz and Coleman, 2012; Zhang et al., 2013b). Recent evidence implicates that TRAF4 is a critical molecule of AKT activation in human cancers. For example, TRAF4 overexpression facilitates cell migration, invasion and the process of epithelial-mesenchymal transition (EMT) via activation of PI3K/AKT signaling in hepatocellular carcinoma (Liu et al., 2017). TRAF4-induced cell proliferation and invasion are dramatically attenuated by blocking AKT with its specific siRNA in osteosarcoma cell (Yao et al., 2014). TRAF4 also promotes tumorigenesis through activation of PI3K/AKT in lung cancer and breast cancer (Li et al., 2013; Peng et al., 2014; Zhang et al., 2014a). There are some possibilities behind on this biological progression. Previous study demonstrated that recruitment of AKT to the cell membrane was critical for AKT activation and TRAF4 was required for AKT activation (Li et al., 2013). The IHC results in our study revealed that TRAF4 was both existed in cell cytoplasm and the nucleus, which indicated that TRAF4 might function in activating AKT via moving through the membrane between cell cytoplasm and the nucleus. According to previous studies and our inference, we supposed that TRAF4 may regulate cell survival through PI3K/AKT pathway in EC cells. In line with our expectations, we found that TRAF4 overexpression increased the phosphorylation of AKT and PI3K in EC cells and tissues, and the inhibitor of PI3K/AKT signaling, LY294002, blocked the stimulative effect of TRAF overexpression on the pathway.

In similar with previous report (Teng et al., 2014), we also found that Oct4 is a downstream factor of PI3K/AKT signaling pathway in EC. Oct4 expression has the potential in inducing activation of AKT. In previous report, study demonstrated that overexpression of Oct4 induced TCL1 expression which further activated expression of AKT (Wang et al., 2010). While, as we concerned, TRAF4 could activate PI3K/AKT pathway. Therefore, there might be a cascade reaction between TRAF1-PI3K/AKT-Oct4. Interestingly, in our study, we found that Oct4 expression was positively correlated with the level of TRAF4 in EC, and TRAF4 increased cell viability through activation of PI3K/AKT/Oct signaling pathway, which suggested that TRAF4 might play important roles in regulating cell growth.

5. Conclusion

In conclusion, the present study demonstrated that TRAF4 promoted cell growth and migration in EC and the mechanism might be through activation of PI3K/AKT/Oct signaling pathway. To the best of our knowledge, this is the first study to demonstrate that TRAF4 may play an oncogenic role in EC and Oct4 is regulated by TRAF4. It may provide us a new insight that TRAF4 might act as a promising molecular target for human EC prevention and treatment in the future.

Conflict of interest

The authors declare that they have no conflicts of interest with the contents of this article.

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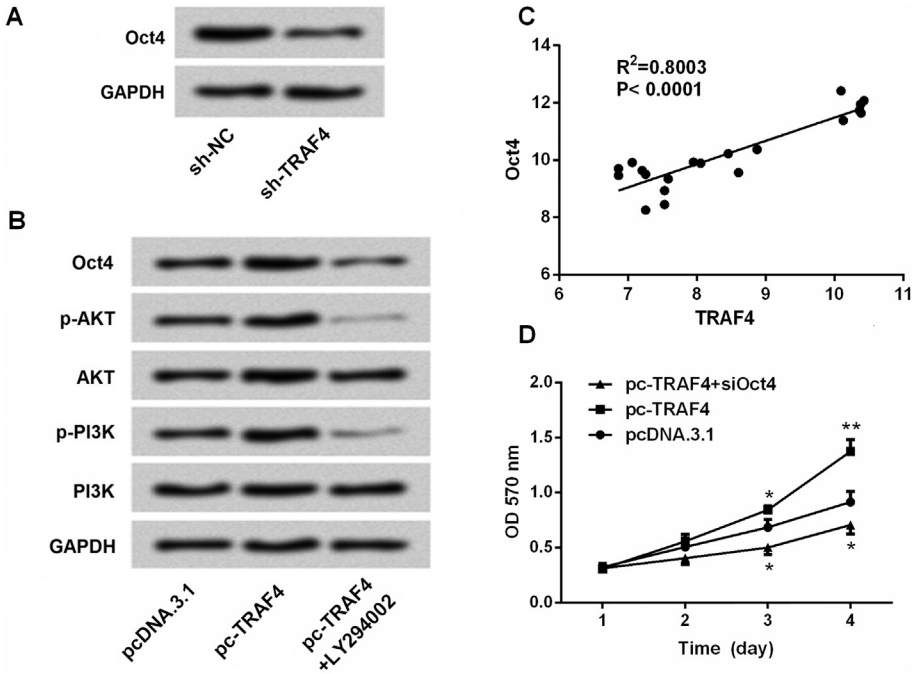

Fig. 6. TRAF4 increased cell viability through regulation of PI3K/AKT/Oct4 pathway. Ishikawa cells were transfected with sh-TRAF4 or pc-TRAF4, or pc-TRAF4 plus Oct4 siRNA. (A) Western blot analysis showed that TRAF4 inhibition reduced the expression of Oct4. (B) LY294002 was applied to inhibit PI3K/AKT pathway. The protein level of p-AKT, p-PI3K and Oct4 were increased in pc-TRAF4 group, while reduced in pc-TRAF4 + LY294002 group. (C) Oct4 expression was positively correlated with the level of TRAF4 in EC tissues (n = 20). (D) TRAF4 overexpression significantly increased cell viability after transfection for 3 day, and the effect of TRAF4 overexpression on cell viability was reversed by Otc4 inhibition by siRNA (n = 3). * $P < .05$, ** $P < .01$.

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None.

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