Cancer Letters 452 (2019) 1–13

Contents lists available at ScienceDirect

Cancer Letters

journal homepage: www.elsevier.com/locate/canlet

Original Articles

TRIP4 promotes tumor growth and metastasis and regulates radiosensitivity of cervical cancer by activating MAPK, PI3K/AKT, and hTERT signaling

Yilin Che[a,1], Yizhuo Li[b,1], Fufu Zheng[c,1], Kun Zou[d,1], Zongjuan Li[a], Manyu Chen[e], Sheng Hu[e], Chunfang Tian[e], Wendan Yu[e], Wei Guo[e], Meihua Luo[f], Wuguo Deng[b,**], Lijuan Zou[a,*]

[a] The Second Affiliated Hospital of Dalian Medical University, Dalian, China
[b] Sun Yat-sen University Cancer Center, State Key Laboratory of Oncology in South China, Guangzhou, China
[c] The First Affiliated Hospital of Sun Yat-sen University, Guangzhou, China
[d] The First Affiliated Hospital of Dalian Medical University, Dalian, China
[e] Institute of Cancer Stem Cell, Dalian Medical University, Dalian, China
[f] The Shude Hospital, Southern Medical University, Foshan, China

ARTICLE INFO

Keywords:
TRIP4
Cervical cancer
Radioresistance
AKT/MAPK
Telomerase

ABSTRACT

Thyroid hormone receptor interactor 4 (TRIP4), a subunit of the tetrameric nuclear activating signal co-integrator 1 (ASC-1) complex, exerts pro-tumorigenic effects. The role for TRIP4 in the regulation of cervical cancer growth and radiation resistance is presently unknown. In this study, TRIP4 was found to be highly expressed in cervical cancer cells and tumor tissues. Knockdown of TRIP4 significantly suppressed cervical cancer cell proliferation and epithelial-mesenchymal transition (EMT), accompanied by inactivation of PI3K/AKT and MAPK/ERK signaling. TRIP4 was also found to target hTERT signaling by regulating its binding to the hTERT promoter. Moreover, the knockdown of TRIP4 increased cell sensitivity to radiation, concomitant with downregulation of Rad51 and p-H2AX. We also demonstrated in an *in vivo* study that the knockdown of TRIP4 effectively suppressed cervical cancer growth and progression in a xenograft tumor model, and these effects were concomitant with the downregulation of p-AKT, p-ERK, p-MEK1/2, MMP-9 and hTERT expression. Immunohistochemical analysis of tumor tissue microarrays showed that TRIP4 overexpression predicted poor prognosis in patients with cervical cancer. Collectively, these results show that TRIP4 plays an essential role in cervical cancer growth and survival.

1. Introduction

Cervical cancer involves uncontrolled cell division and tissue invasion of the female uterine cervix, and it continues to be one of the most common cancers among women [1]. Although the control of human papillomavirus (HPV) infection and early screening can effectively reduce the incidence of cervical cancer, gene mutations still play a key role in its development. Investigators have recently begun to view these mutations as biomarkers to determine disease progression and for use as therapeutic targets [2]. In recent years, treatment strategies for cervical cancer have been proposed that include small molecular inhibitors, monoclonal antibodies, and macromolecules or nucleic acids as therapeutic modalities. These new methodologies must be able to effectively prevent the interaction between viral HPV proteins and their cellular targets. Although some results show promise [3], there is still a need to develop new therapeutic targets and treatment options.

TRIP4 is a subunit of the transcriptional coactivator ASC-1. ASC-1 is responsible for bridging transcription factors or remodeling the chromatin structure [4], and it was initially isolated as a transcription factor and protein partner of the thyroid hormone receptor (THR) [5]. Recent studies have indicated that ASC-1 can play a role in the development of tumors, and it acts as a coactivator of the transcription factors AP1, SRF, and NF-κB in HeLa cells [6]. It has also been demonstrated that ASC-1 localizes to the nucleus and cytoplasm of rat fibroblasts and regulates the expression of the anti-apoptotic molecule plasminogen activator inhibitor-2 (PAI2) in gastric cancer cells under different conditions [7]. A recent report indicated that ASC-1 promotes the progression of melanomas by regulating the expression of COX-2/iNOS

* Corresponding author.
** Corresponding author.
E-mail addresses: dengwg@sysucc.org.cn (W. Deng), lijuanzou1963@163.com (L. Zou).
[1] These authors contributed equally to this work.

https://doi.org/10.1016/j.canlet.2019.03.017
Received 20 November 2018; Received in revised form 8 March 2019; Accepted 18 March 2019
0304-3835/

[8]. However, the functions and mechanism(s) underlying TRIP4's effects in cervical cancer are not fully understood.

Telomerase is a nuclear ribonucleoprotein protease complex that may control the immortalization of cells, and its activity may be related to cellular senescence [9]. Telomerase itself carries a template and maintains telomere length by repeatedly synthesizing telomeric DNA [10]. Telomerase activity in human cancer cells is therefore much higher than in most human normal cells [11]. It has been reported that hTERT plays an important role in tumorigenesis, growth, migration, and invasion [12], and its inhibition significantly reduces telomerase activity and telomere length, thereby attenuating the growth of cervical cancer tumors, cellular proliferation, cell migration, and invasion *in vitro* [13]. Therefore, the regulation of telomerase activation and its expression levels remains an area that requires further exploration.

In the present study, we examined the effects of TRIP4 on cervical cancer cell proliferation, and radioresistance, and we further identified the underlying molecular mechanisms *in vitro* and *in vivo*. We also assessed whether TRIP4 expression was correlated with overall survival in cervical cancer. Our results showed that the knockdown of TRIP4 led to reduced cell proliferation, invasion, and migration, and it increased radiosensitivity in cervical cancer cell lines. Such proliferation-inhibiting effects of TRIP4 knockdown were associated with inactivation of the MAPK and PI3K/AKT pathways and inhibition of the hTERT signaling pathway. Our findings show novel potential mechanisms in the development of cervical cancer and provide promising new therapeutic targets.

2. Materials and methods

2.1. Cell lines and culture

We obtained the human cervical cancer cell lines HeLa, SiHa, C33-A, DoTc2, HeLa S3, and Caski, and normal cervical cells Ect1 from the American Type Culture Collection (ATCC). All of the cells were cultured in a modified medium supplemented with 10% fetal bovine serum (FBS), 100 μg/mL penicillin, and 100 μg/mL streptomycin. HeLa, SiHa, and C33-A cells were specifically cultured in Eagle's minimal essential medium (EMEM); and DoTc2, HeLa S3, and CaSki cells in Dulbecco-modified Eagle medium (DMEM). Cells were maintained in an incubator at 37 ˚C, 5% CO_2 in compressed air at high humidity.

2.2. siRNA design and transfection

To inhibit the expression of TRIP4, cells were transfected with TRIP4-specific siRNAs (siRNA1, 5′-GCCACUGACCAAAUUGGAUTT-3′, 5′-AUCCAAUUUGGUCAGUGGCTT-3'; siRNA2, 5′-GGACUAGAGUUCA ACUCAUTT-3′, 5′- AUGAGUUGAACUCUAGUCCTT-3′) or non-specific siRNA (10 μmol/L) synthesized by Shanghai GenePharma Company (Shanghai, China). Cells plated in 96-well plates (5000 cells/well) or six-well plates (200,000 cells/well) were transfected with siRNA duplexes (1–2 μg) encapsulated by Lipofectamine 3000 (Invitrogen, Carlsbad, CA). At 48 h after treatment, protein expression and cell viability were tested by western blot and MTT analysis, respectively.

2.3. Plasmid vectors and transfection

The TRIP4 plasmid was purchased from Origene. TRIP4 plasmids with different lengths of gene sequence were constructed in our lab and used in the transfection experiments. The transfection was performed using Lipofectamine 3000 reagent according to the manufacturer's protocol. To overexpress TRIP4 in HeLa or SiHa cells, cells were transfected with control vector LacZ or TRIP4-overexpressing plasmids.

2.4. Establish a stable cell line

TRIP4-targeting shRNAs for knockdown of its expression in HeLa cells. Four specific shRNA plasmids and a control non-targeting shRNA plasmid fused to mCherry were purchased from Gene Copoeia (Rockville, USA). Virus packaging and transfection were performed according to the Lenti-Pac™ HIV Expression Packaging Kit User Manual (Gene Copoeia). Finally, the cell lines SH1 (5′-GGTCAATTCATAGAAG AACTT-3′) and SH2 (5′-GGTAAATCCCAACATGTACCA-3′) with higher efficiency of TRIP4 silencing were selected by fluorescence microscope and Western blot analysis. Similarly, HeLa or SiHa cells were infected with lentivirus (LvOE) to upregulate cellular TRIP4.

2.5. Western immunoblotting analysis

We separated proteins on a 10% SDS-PAGE gel and electrophoretically transferred them to a PVDF membrane. The proteins were probed with antibodies (1:1,000) to P85, p-P85, AKT, p-AKT, p110γ, p-Raf, MEK1/2, p-MEK1/2, P38, p-P38, ERK, p-ERK, Slug, Snail, Rad51, N-cadherin, and p-H2AX (Cell Signaling Technology); TRIP4, hTERT, MMP1, and MMP9 (Santa Cruz Biotechnology); β-catenin, E-cadherin and β-actin (Proteintech); and TFIIB (Abcam) for 12 h at 4 ˚C, followed by incubation with HRP-conjugated secondary antibody for 2 h. Detection was by chemiluminescence (Bio-Rad Laboratories, Inc., USA).

2.6. Cellular viability assay

We determined cell viability by MTT assay (Roche Diagnosis, Indianapolis, IN). Cells were added to a 96-well plate (3000 cells/well) and cultured overnight, then continuously cultured in a medium free of FBS. Six hours later, cells were transfected with siRNA or plasmid. After an additional 48 h of continuous culture, cellular viability was determined.

2.7. Colony formation assay

We treated cervical cancer cells with TRIP4 siRNA or lentivirus overexpressing TRIP4. The cell culture medium was changed 12 h after transfection, and cells were harvested after 48 h of cell culture. Cells were seeded in a 6-well plate (600 cells/well) and incubated for 12 days. We treated colonies with 0.1% crystal violet at room temperature for 15 min, then counted and photographed cell colonies containing more than 50 cells.

2.8. Immunofluorescence

HeLa cells were fixed with 4% paraformaldehyde for 30 min after knockdown with TRIP4 siRNA, and placed in 0.2% Triton X-100 in PBS solution to permeabilize for 5 min. Bovine serum albumin (BSA, 10%) was added to cells for 20 min, then they were incubated with primary antibodies (1:200) against vimentin or E-cadherin for 12 h. After washing with PBS containing 1% BSA, the corresponding secondary antibody labeled with fluorescein was added to cells for 1 h. Protein localization was analyzed using a confocal microscope (Leica) and processed using Image-Pro Plus 5.1 software (Media Cybernetics, Inc.).

2.9. Scratch assay

HeLa and SiHa cells were grown to 80–90% confluency in a 6-well plate, and a scratch assay was performed to examine cellular migration. We damaged the cellular monolayer with the tip of a sterile 100-μL pipette and washed it with PBS to remove isolated cells. After transfection with TRIP4 siRNA for 8 h, we photographed the wound space at 0 and 48 h with a Leica DM 14000B microscope, and recorded and calculated the distance of the wound gap using ImageJ v1.49 (NIH, USA). The migration rate was calculated as MR = (initial gap (0 h) - terminal gap (48 h)/initial gap (0 h)) × 100%. The experiment was repeated three times and the data presented as the mean ± SD (*$p < 0.05$).

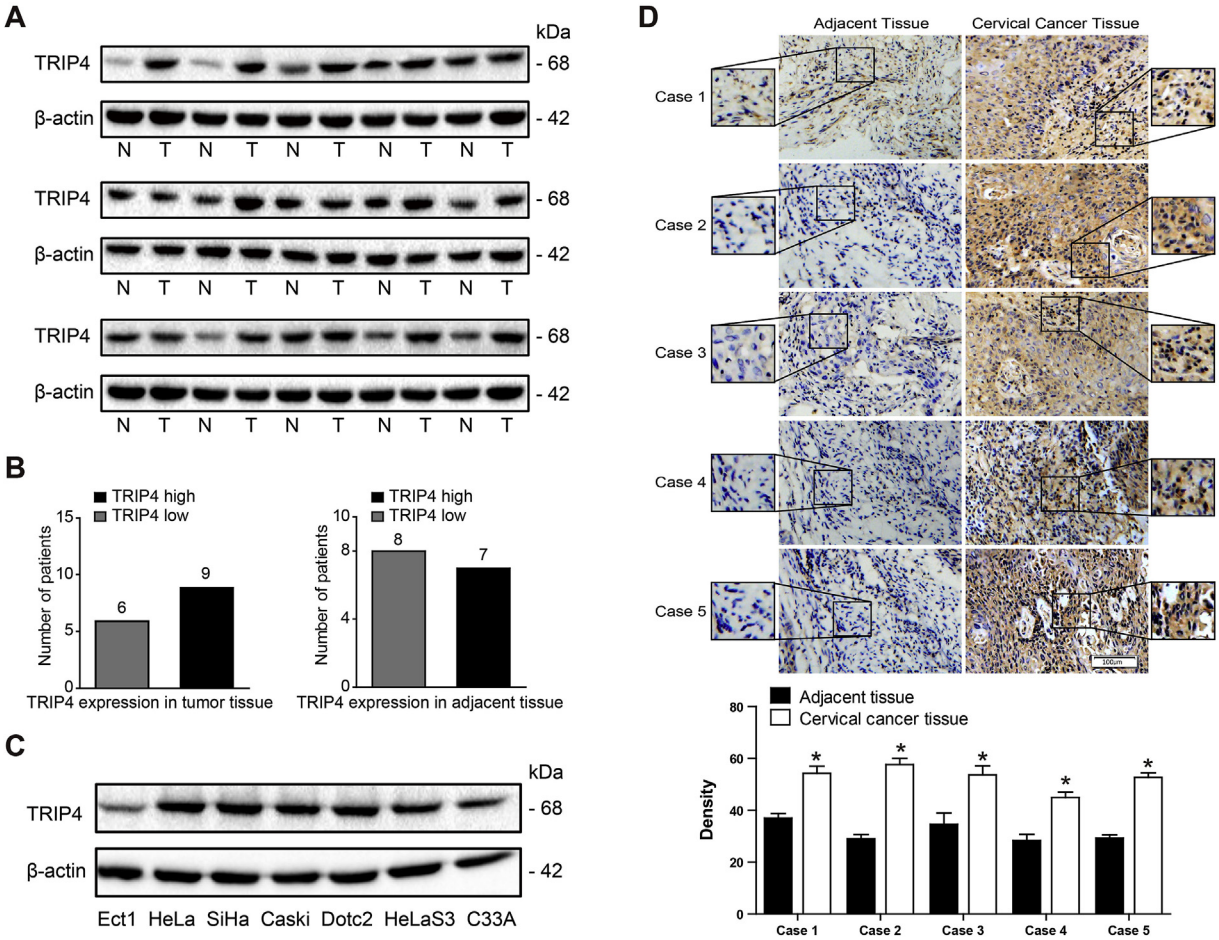


Fig. 1. TRIP4 was highly expressed in cervical cancer cell lines and tumor tissues. (A) Proteins were extracted from cancer tissues and adjacent normal tissues of 15 cervical cancer patients, and the expression of TRIP4 was detected by Western blot (n = 15). Data represent the mean ± SD of three independent experiments. *p < 0.05 vs. control. **(B)** The distribution of cervical cancer patients with TRIP4 expression in tumor tissues and adjacent tissues (n = 15). **(C)** The expression of TRIP4 protein in a normal cervical cell line and various cervical cancer cell lines was examined by Western blot analysis. **(D)** The expression of TRIP4 protein in tumor tissues from cervical cancer patients and corresponding adjacent tissues was examined by immunohistochemical analysis (n = 5; × 40 magnification). The data represent the mean ± S.D. of three independent experiments. The level of significance is indicated by *P < 0.05.

2.10. Transwell assay

We used the transwell assay to determine the invasive abilities of HeLa and SiHa cells. The 24-well plates contained 500 µl of DMEM supplemented with 20% FBS per well. The upper chamber (Cat: 3422, Corning) was then coated with 20 µl (1 mg/ml) of Matrigel matrix (Cat: 356234, Corning). The control and TRIP siRNA cells were each re-suspended in 100 µl of DMEM without FBS. We placed the chambers into 24-well plates and added 3×10^6 cells to each chamber for 24 h of culture. Cells at the bottom of the upper chamber were fixed with 4% paraformaldehyde for 5 min and stained with 0.1% crystal violet for 10 min, then we acquired photomicrographic images with an Olympus microscope (200× magnification, Olympus Corp.)

2.11. Radiation assay

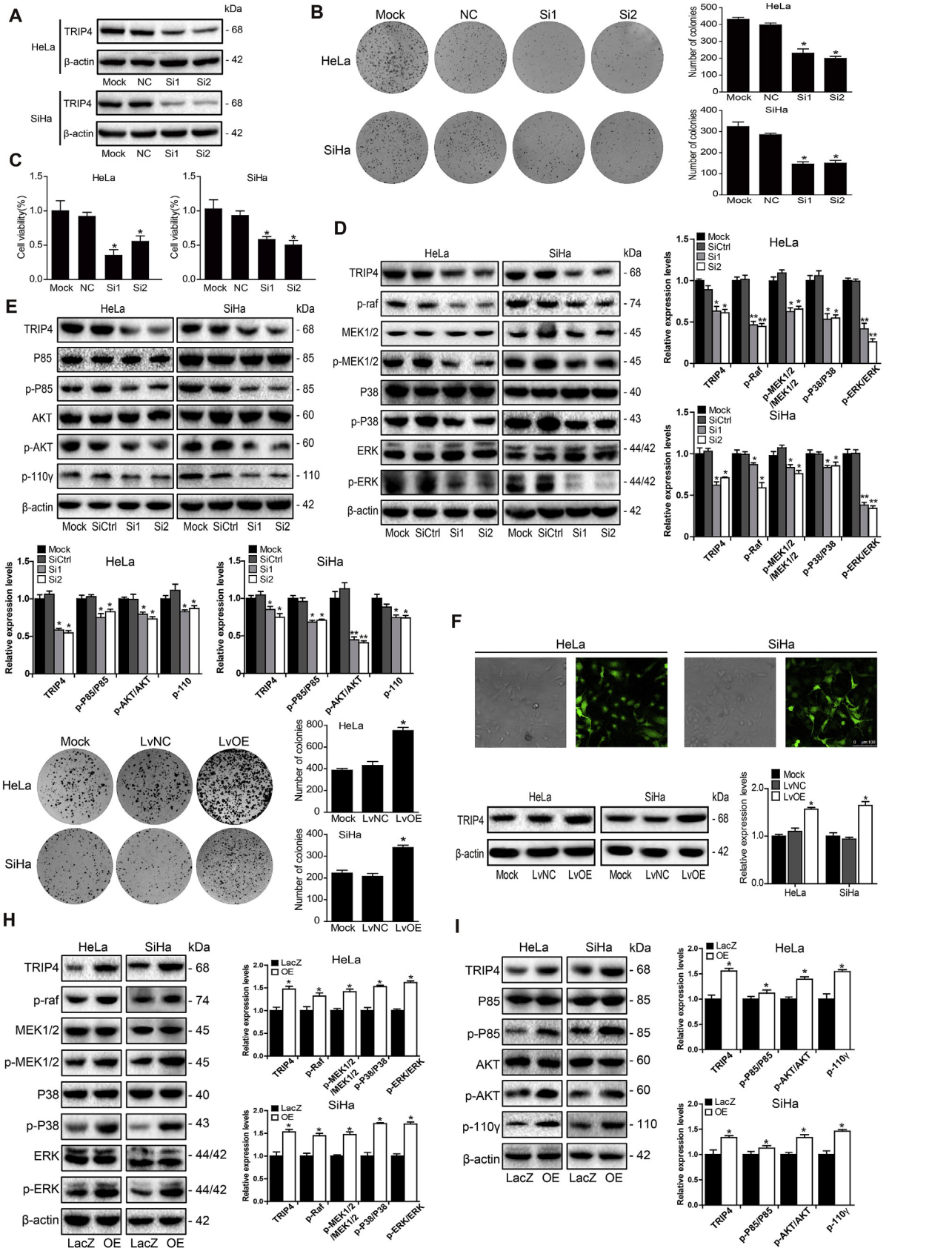
X-ray irradiation was applied using an X-320ix (Precision X-Ray, Inc., North Branford, CO, USA). Cell Irradiation: Cells were irradiated with a single dose of 4 Gy of 6 MV X-rays. The cells were covered with 1.0 cm equivalent of wax plate and irradiated under air. Mouse irradiation: The mice were anesthetized and placed in a glass box for fixation. The tumor was located in the center of the irradiation field and was irradiated with 6 MV X-rays at a dose of 4 Gy/exposure, once daily on Day 1, Day 3, Day 5 and Day 7 with a total dose of 16 Gy.

2.12. Tumor cell comet assay

Following the indicated treatments, we resuspended the collected cervical cancer cells and then loaded them onto agarose-coated slides (150 µl of 0.5% agarose at a density of 1.5×10^3 cells/µl). The slides were then immersed in lysis buffer (10 mM Tris-HCl, 2.5 M NaCl, 100 mM EDTA, 1% Triton X-100, and 10% DMSO) for 1 h and washed with neutralization buffer for 5 min, 3 times each. The slides were then placed in cold electrophoresis solution (300 mM NaOH and 1 mM EDTA) and subjected to 25 V for 25 min under electrophoresis at 300 mA. We stained the cells on the slides with ethidium bromide solution (20 µg/ml) and captured the images using an Olympus fluorescence microscope (Olympus Corp.). The number of cells with or without the appendix was calculated and averaged by Comet Assay Software Project (CASP) (percentage DNA in tail = 100 − head fluorescent intensity/(head fluorescent intensity + tail fluorescent intensity) × 100Tail length = full length - head length), and experiments were repeated 3 times.

2.13. Nuclear extraction

We lysed cells in 250 µL of cytoplasm lysis buffer (10 mM HEPES [pH 7.9], 10 mM KCl, 1.5 mM $MgCl_2{\cdot}6H_2O$, 300 mM of sucrose, and 0.5% NP-40) and mixed the buffer with protease inhibitors (10 mM NaF, 2.5 mM β-glycerophosphate, 1 mM Na_3VO_4, 0.1 mM PMSF, 1 g/



(caption on next page)

Fig. 2. TRIP4 promoted cervical cancer cell growth and survival *in vitro* concomitant with activation of MAPK and PI3K/AKT signaling pathways. (A) TRIP4 expression in HeLa cells and SiHa cells transfected with TRIP4-specific siRNA (si-TRIP4-1 and si-TRIP4-2) was analyzed by Western blot. **(B)** Colony formation assay of HeLa and SiHa cells transfected with TRIP4 siRNA or nonspecific control siRNA. Colonies (> 50 μm) were counted after 12 days. Data represent the mean ± SD of three independent experiments. *p < 0.05 vs. control. **(C)** Cell viability was measured in HeLa cells and SiHa cells by MTT assay after TRIP4 knockdown. **(D, E)** The proteins in the MAPK pathway or PI3K/AKT pathway of HeLa and SiHa cells were analyzed by Western blot 48 h after transfection. Data represent the mean ± SD of three independent experiments. *p < 0.05 and **p < 0.01 vs. control. **(F)** HeLa and SiHa cells transfected with TRIP4-overexpressing lentivirus (LvOE) were monitored under a fluorescent microscope. The overexpression of TRIP4 in cervical cancer cells HeLa and SiHa was detected by Western blot. **(G)** The number of cell colonies in HeLa and SiHa cell lines was determined by colony formation assay. Data represent the mean ± SD of three independent experiments. *p < 0.05 vs. control. **(H, I)** After the cells were transfected with TRIP4 siRNA or overexpressing plasmid, the proteins in the MAPK pathway or PI3K/AKT pathway of HeLa and SiHa cells were analyzed by Western blot 48 h after transfection. Data represent the mean ± SD of three independent experiments. *p < 0.05 vs. control.

mL of leupeptin, and 0.5 mM dithiothreitol) for 10 min. We then centrifuged the mixture at 2600 × *g* for 1 min at 4 °C. The pellet was resuspended in 70 μL of nuclear lysis buffer (20 mM HEPES [pH 7.9], 420 mM NaCl, 1.5 mM $MgCl_2 \cdot 6H_2O$, 0.1 mM EDTA, and 2.5% glycerol) with various protease inhibitors and placed on ice for 10 min. The mixture was centrifuged at 10,400 × *g* for 10 min at 4 °C, and the nuclear protein concentration was determined by BCA assay.

2.14. Promoter reporters and luciferase assay

Cells (2×10^5 cells/well) were seeded in a 6-well plate, and then transfected with hTERT promoter luciferase plasmids or GFP reporter vectors (driven by a CMV or an hTERT promoter) with Lipofectamine 3000. We analyzed the luciferase activity using the Luciferase reporter assay system Enspire2300 (Perkin Elmer).

2.15. Tissue microarrays

Cervical cancer tissue microarrays were obtained from Outdo Biotech (Shanghai, China). The tumor tissue samples (cancers and adjacent non-cancers) were took from patients who had not undergone anti-tumor therapy since diagnosis, with all of the information from patients authenticated. The expression levels of TRIP4 and hTERT proteins were analyzed based on the staining levels of the tissue microarrays. We scored the slides based upon the amount of staining.

2.16. Chromatin immunoprecipitation (ChIP) assay

The cells were fixed with 1% formaldehyde for 10 min at room temperature. The redundant formaldehyde was cross-linked with 0.125M glycine for another 10 min. Then the cells were washed with 1 × PBS twice, scraped, collected, and resuspended in SDS lysis buffer (1% SDS, 10 mM Tris-HCl, PH8.0). Then the cells were sonicated on ice to cleave the intact DNA into 100–1000 bp fragments. A small portion of the cell lysate was used as a DNA input control, and the remaining total lysate was divided into two portions, incubated respectively with anti-TRIP4 antibody or non-immune rabbit IgG (Proteintech Company, USA). The complex of antibody-chromatin were immunoprecipitated by protein A/G plus agarose beads. The protein/DNA complexes were washed with elution buffer (20% SDS, 1M NaHCO3), reversely cross-linked at 65 °C and extracted by phenol/chloroform. The extracted DNA was subjected to PCR to amplify a 220 bp region (−378 to −159 bp) of the hTERT promoter using primers (sense, 5′-ACC CTG GGA GCG CGA GCG GC-3'; antisense, 5′-GGG GCG GGG TCC GCG CGG AG-3′). The PCR products were resolved electrophoretically on a 1% agarose gel and visualized by ethidium bromide staining.

2.17. Pulldown assay

A biotin-labeled double-stranded DNA probe corresponding to the −378 to −159 bp nucleotides of the hTERT promoter sequence was synthesized by PCR (sense, 5′-ACC CTG GGA GCG CGA GCG GC-3'; antisense, 5′- GG GCG GGG TCC GCG CGG AG-3′). Then, 400 μg nucleoprotein extracts, 4 μg DNA probe, and 50 μl streptavidin agarose beads (Sigma-Aldrich) were mixed in 500 μl prepared PBSI buffer containing 0.5 mM PMSF, 10 mM NaF, 25 mM β-glycerophosphate and incubated on a rotary shaker for 2 h at room temperature, and then the DNA-protein complex was precipitated by centrifugation, washed with PBSI buffer twice, and then were resuspended with loading buffer and boiled at 100 °C for 10 min. Western blotting was used to analyze the protein in the complex.

2.18. Immunohistochemical staining

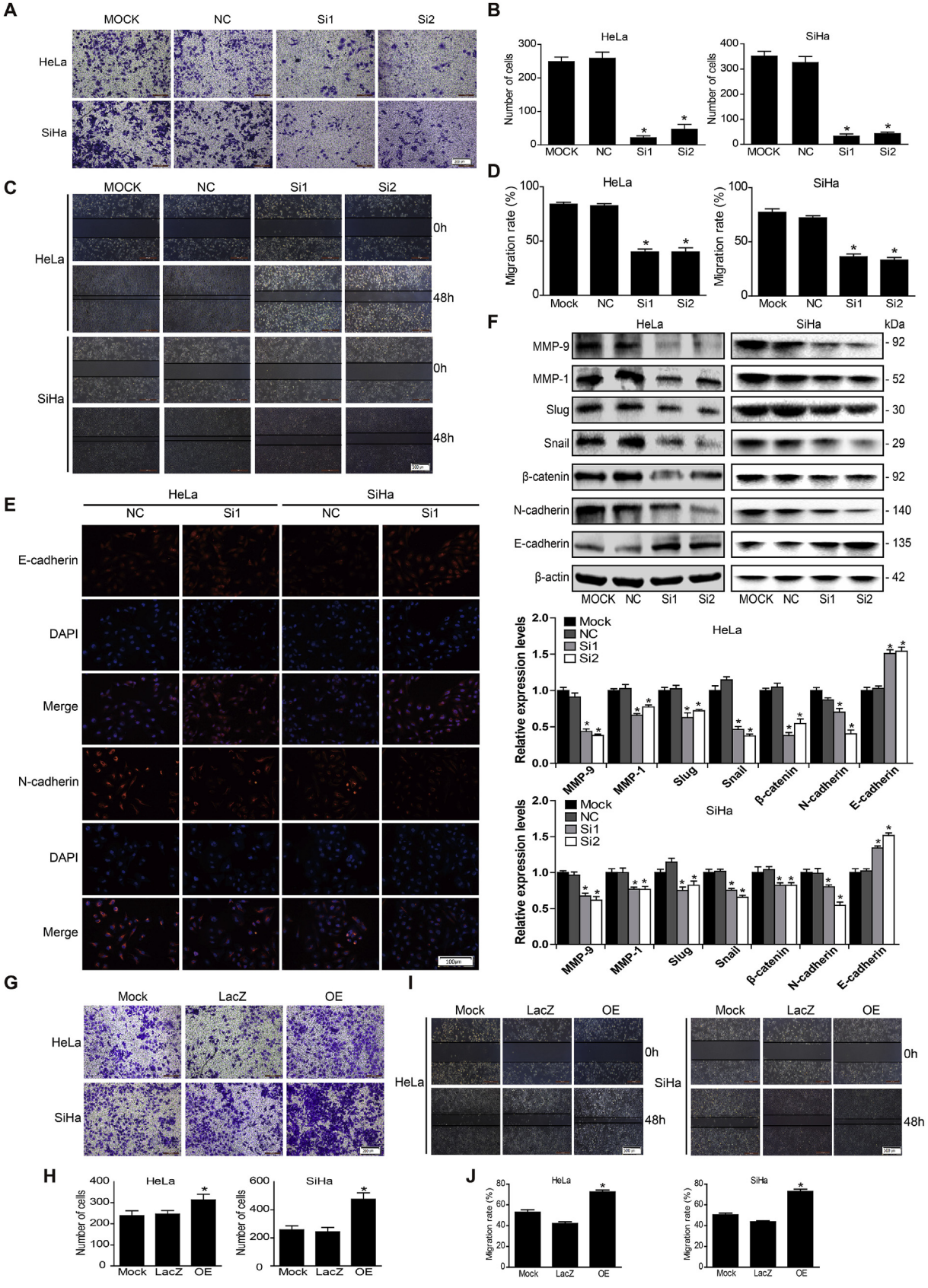
Immunohistochemical (IHC) staining was performed according to the DAB (3, 3′-diaminobenzidine) Kit (Origene, China) and applied to cervical cancer tissues, tissue microarrays, and nude mice. We applied primary antibody to each of the glass-coated samples and incubated them for 12 h at 4 °C (TRIP 4, Santa Cruz, 1:50; p-AKT, p-ERK, and p-H2AX, Cell Signaling Technology, 1:150; hTERT, Santa Cruz, 1:50). The second antibody with Streptavidin/Peroxidase was added according to the manufacturer's protocol. Then the slides were counterstained in hematoxylin after development with DAB. For each specimen, the total score of expression intensity (negative staining: 0; weak staining: 1; moderate staining: 2; and strong staining: 3) was multiplied by the stained cell number (positive cells as ≤25% of the cells, 1; 26–50% of the cells, 2; 51–75% of the cells, 3; > 75% of the cells, 4). When the sample was scored ≥7, we defined it as high expression, otherwise low expression.

2.19. Development of in vivo tumor model and tissue processing

Four-week-old female BALBc/nu mice were purchased by the Animal Center of Dalian Medical University, and all animal maintenance and procedures were carried out in accordance with the National Institute of Health Guide for the Care and Use of Laboratory Animals, and passed through the training process and was approved by the Animal Care and Ethics Committee of Dalian Medical University. Four groups (Mock, ShNC, Sh1, and Sh2) of HeLa cells (4×10^6 cells, resuspended in cold PBS) were injected subcutaneously into the left iliac skin. Tumor sizes and body weights of nude mice were recorded on day 4, and the experiment was terminated 20 days after tumor cell inoculation. Tumor size was measured every 4 days using a Vernier caliper. Upon completion of the experiment, we killed the mice, the tumors from each mouse were excised, and weights were calculated for statistical analyses. Then tumor samples were fixed with 10% formalin and embedded in paraffin for further TRIP4, hTERT, p-AKT, p-ERK, and p-H2AX expression analysis by western blotting and IHC staining carried out as described above. A portion of each tumor was prepared for immunohistochemical staining with TRIP4 (1:50), hTERT (1:50), p-AKT (1:150), p-ERK (1:150), or p-H2AX (1:150). Another portion of the tumor was prepared for Western blot analysis.

2.20. Statistical analysis

Each experiment was repeated 3 times under the same conditions. The results are shown as means ± standard deviation (SD) and were statistically analyzed using GraphPad Prism software version 5.01



(caption on next page)

Fig. 3. TRIP4 promoted the migration and invasion of cervical cancer cells. (A) HeLa and SiHa cells transfected by TRIP4 siRNA1 or siRNA2 were planted in a chamber covered with a diluted Matrigel matrix. The invading cells were stained and observed at × 40 magnification. **(B)** Quantitative analysis of invading cells in A. *P < 0.05. **(C)** Cell migration was analyzed by scratch assay. HeLa and SiHa cells were seeded in 6-well plates and wounded by a 100 μl yellow pipette tip. Then the cells were grown for 48 h for photo recording. **(D)** Cell migration rate in C was measured and calculated. The data are presented as the mean ± SD of three separate experiments. (*P < 0.05). **(E)** TRIP4-knockdown human cervical cancer cells (HeLa and SiHa) grown on chamber slides were cultured for 24 h, and subcellular localization of E-cadherin and N-cadherin, was examined by confocal microscopy. **(F)** The levels of MMP-9, MMP-1, Slug, Snail, β-catenin N-cadherin and E-cadherin proteins in HeLa and SiHa cells were analyzed by Western blot. **(G, I)** Transwell and scratch assays were performed to detect the invasion ability of TRIP4 HeLa and SiHa cells transfected with a TRIP4-overexpression plasmid, and the invading cells were stained and observed at × 40 magnification. **(H, J)** Quantitative analysis of invading cells. Data represent the mean ± SD of three independent experiments. *p < 0.05 vs. control. (For interpretation of the references to colour in this figure legend, the reader is referred to the Web version of this article.)

(GraphPad Software, Inc., La Jolla, CA, USA). Student's *t*-test was used to compare the values of the test and control samples, and a P value of less than 0.05 or 0.01 was considered to be statistically significant.

3. Results

3.1. TRIP4 is highly expressed in cervical cancer cell lines and tumor tissues

We first examined by Western immunoblotting the expression of TRIP4 protein in the tumorous and paracancerous tissues from 15 cervical cancer patients (Fig. 1A and B), in the normal human cervical cell line Ect1, and in 6 cervical cancer cell lines (HeLa, SiHa, Caski, DoTc2, HeLa S3, and C33A) (Fig. 1C). We also detected TRIP4 protein expression in cervical cancer tissues and paracancerous tissues from 5 different patients by IHC (Fig. 1D). The results showed that TRIP4 exhibited higher expression in tumor cell lines or cervical cancer tumor tissues compared to their corresponding adjacent normal cells or normal tissues. These results suggest that TRIP4 may be a potential biomarker for human cervical cancer.

3.2. TRIP4 promotes cervical cancer cell growth in vitro and alters MAPK and PI3K/AKT signaling pathways

To study the biological function of TRIP4, we chose the 2 cervical cancer cell lines, HeLa and SiHa, as our cellular models. Non-specific control siRNA (NC) and TRIP4-siRNA (Si1 and Si2) were transfected into HeLa and SiHa cells, and after 48 h of treatment, the expression of TRIP4 protein, cellular proliferation, and clonal formation were determined. The results showed that knockdown of TRIP4 effectively downregulated TRIP4 protein expression in both cell lines (Fig. 2A). Moreover, the knockdown of TRIP4 also significantly inhibited cellular clone formation (Fig. 2B) and viability (Fig. 2C) compared with the control group transfected with non-specific siRNA (NC). To determine the potential molecular mechanism(s) governing the ability of TRIP4 to promote the survival of cervical cancer cells, we detected by immunoblotting assay several pro-survival proteins that may be affected by TRIP4. Our results showed that knockdown of TRIP4 in HeLa cells and SiHa cells inhibited the expression of the phosphorylated forms of PI3K (p85 and p110γ), AKT, Raf, MEK1/2, p38, and ERK, but it barely affected total PI3K, AKT, MEK1/2, p38, and ERK protein levels (Fig. 2D and E).

When we treated the HeLa and SiHa cell lines with TRIP4 overexpressing lentivirus (Fig. 2F), we found that cellular clone formation was enhanced (Fig. 2G). Moreover, HeLa and SiHa cells treated with TRIP4 overexpression plasmid increased the levels of the phosphorylated PI3K, AKT, MEK1/2, p38, and ERK protein (Fig. 2H and I), suggesting that PI3K/AKT may be involved in TRIP4-mediated growth promotion of cervical cancer in the MAPK/ERK signaling pathway.

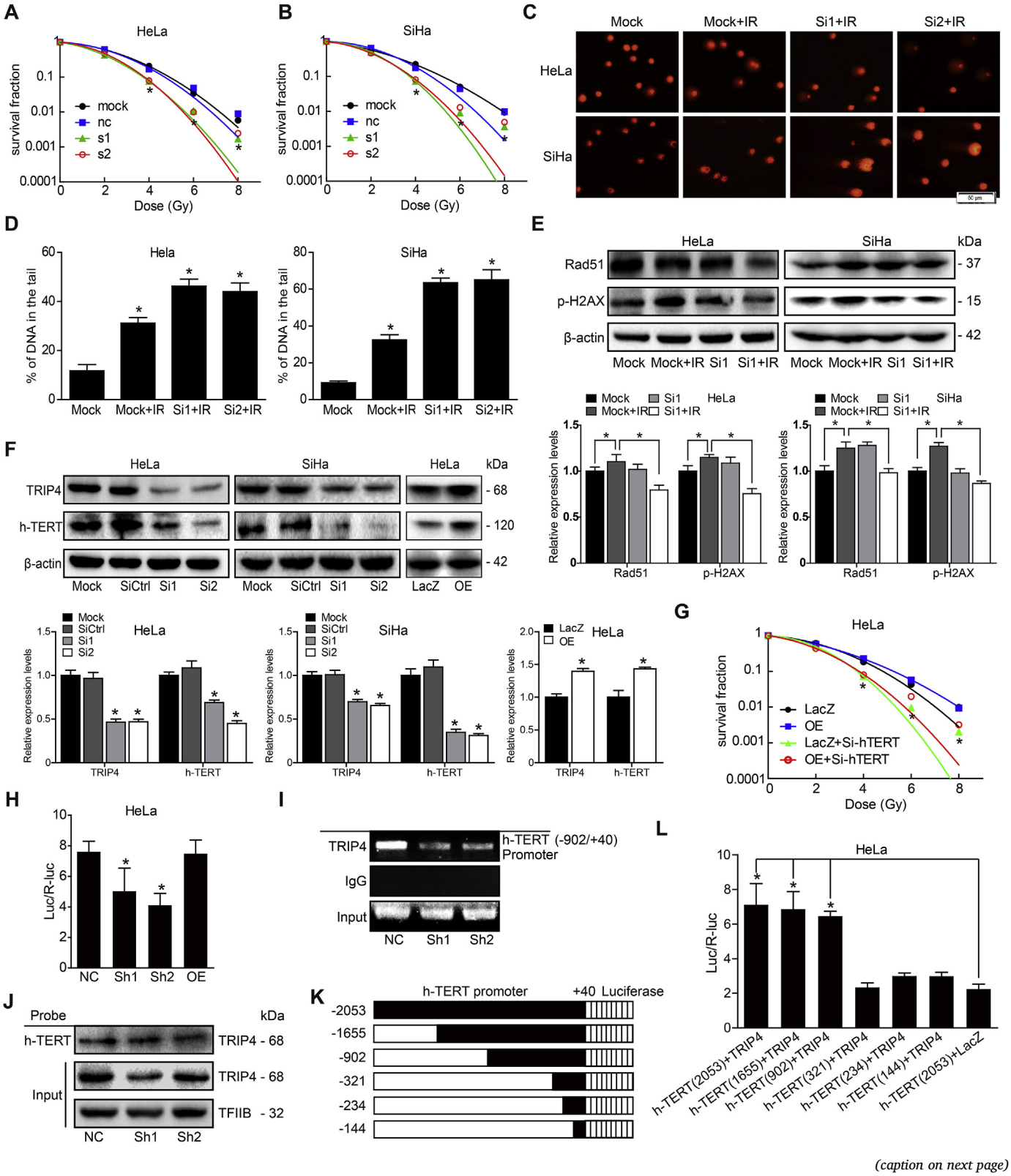
3.3. TRIP4 promotes the migration and invasion of cervical cancer cells

Since the knockdown of TRIP4 decreased the growth and survival of cervical cancer cells, we proposed that a role for TRIP4 in promoting cervical cancer was the acceleration of tumor cell migration and invasion. When we performed Transwell (Fig. 3A and B) and wound healing assays in HeLa and SiHa cells (Fig. 3C and D), we observed that the inhibition of TRIP4 by its specific siRNA decreased the migration ability of cervical cancer cells. We also analyzed migration markers by immunofluorescence and found increased expression of E-cadherin and decreased expression of N-cadherin after TRIP4 knockdown (Fig. 3E). Upon analysis of migration-related molecules by Western blotting (Fig. 3F), we found that the knockdown of TRIP4 effectively increased the levels of E-cadherin, but decreased MMP-9, MMP-1, Slug, Snail, β-catenin, and N-cadherin in HeLa and SiHa cells. When we overexpressed TRIP4 in HeLa and SiHa cell lines, we found that the invasion and migration ability of cancer cells was enhanced (Fig. 3G–J). These results showed that TRIP4 promoted the migration and invasion of cervical cancer cells.

3.4. TRIP4 regulates the expression of hTERT to decrease DNA damage and radiosensitivity in cervical cancer cells

We next performed clonogenic survival assays to investigate the impact of TRIP4 on radiosensitivity in the cervical cancer cell lines HeLa and SiHa. The cells were transfected with siRNA-NC, siRNA-1, or siRNA-2 for 48 h prior to irradiation with 0, 2, 4, 6 or 8 Gy. The principal parameters of HeLa and SiHa cells using dose-survival curves were obtained according to the multi-target single-hit model. A dose-dependent radiosensitization by TRIP4 silencing was also observed with sensitizing enhancement ratios (SER) of 1.28 and 1.46 by siRNA-1 and siRNA-2, respectively (Fig. 4A and B). Next, we used the comet assay to examine DNA damage after TRIP4 knockdown in irradiated cervical cancer cells (Fig. 4C). We found that the average of tail intensity (percentage DNA in the tail) was higher in the TRIP4-knockdown groups (Si1 + IR and Si2 + IR) compared to the control irradiated group (Mock + IR) (Fig. 4D). We assessed the changes in p-H2AX and Rad51 proteins by immunoblotting assays (Fig. 4E), and the results showed that they were both reduced with TRIP4 knockdown. The above results indicate that TRIP4 regulates DNA damage induced by radiosensitivity in cervical cancer cells.

Previous studies have shown that there is a close relationship between telomerase and radiosensitivity [14–16], and radiation particularly increases telomerase activity in cancer cells. In addition, telomerase regulates via a post-translational mechanism the PI3K/AKT pathway [17]. To determine the effect of TRIP4 on hTERT signaling, we transfected HeLa and SiHa cell lines with TRIP4 siRNA, and found that TRIP4 knockdown was accompanied by a simultaneous decrease in hTERT expression, reversing when TRIP4 was overexpressed in HeLa cells (Fig. 4F). Dose dependent radiosensitization of normal and TRIP4 overexpressing HeLa cells by si-hTERT transfection was also observed. (Fig. 4G). When the HeLa cells with stable TRIP4 knockdown or overexpression were transfected with a hTERT promoter-driven luciferase plasmid, and luciferase expression was determined 48 h after treatment, we observed that knockdown significantly inhibited hTERT promoter-driven luciferase expression (Fig. 4H). Chromatin immunoprecipitation assays were performed using the hTERT promoter in HeLa cells, and, as shown in Fig. 4I, the hTERT promoter was amplified and less DNA was precipitated in cells containing shRNA against TRIP4, indicating that TRIP4 was bound to the hTERT promoter. We further confirmed the binding of TRIP4 to the hTERT promoter using a DNA-protein pulldown



(caption on next page)

assay. We mixed streptavidin-agarose beads, a biotin-labeled hTERT promoter probe, and nuclear protein from HeLa cells that stably expressed the control vector or TRIP4 shRNA. As shown in Fig. 4J, the TRIP4 protein was detected by Western blotting in the pulldown complexes.

To further confirm the above results, we constructed 6 different lengths of hTERT promoter-driven luciferase reporter vectors (Fig. 4K), and we co-transfected the plasmid expressing TRIP4 and the luciferase reporter driven by the hTERT promoter into HeLa cells. As shown in Fig. 4L, luciferase expression was higher in cells co-transfected with TRIP4 and hTERT (−2053/+40, −1655/+40, and −902/+40) - luciferase plasmids compared with other lengths of hTERT promoter regions, or cells co-transfected with LacZ and TRIP4-luciferase plasmids. This result not only demonstrated that TRIP4 is a transcriptional

Fig. 4. TRIP4 regulates the transcriptional activity and expression of hTERT to decrease DNA damage and radiosensitivity in cervical cancer cells. (A, B) Clone-forming cell survival curves were generated for HeLa and SiHa cells treated with TRIP4 siRNA for 24 h and then exposed to 2, 4, 6 or 8 Gy X-ray irradiation. Survival data was normalized to the unirradiated control cells. The results are expressed as the mean ± SD of at least three independent experiments. *P < 0.05 vs. control. **(C)** HeLa and SiHa cells were transfected with TRIP4 siRNA. After 48 h, the cells were exposed to 4 Gy radiation and collected for the comet assay and Western blot. **(D)** The percentage of DNA in the tail of 50 random cells was calculated. Data represent the mean ± SD of three independent experiments. *p < 0.05 vs. control. **(E)** The levels of Rad51 and p-H2AX proteins in HeLa and SiHa cells were analyzed by Western blot. Data represent the mean ± SD of three independent experiments. *p < 0.05 vs. control. **(F)** HeLa and SiHa cells were transfected with TRIP4-siRNA (si-TRIP4) or TRIP4-expressing plasmid. 48 h after transfection, the expression of TRIP4 and hTERT protein levels were detected by Western blot. Data represent the mean ± SD of three independent experiments. *p < 0.05 vs. control. **(G)** Clone-forming cell survival curves were generated for HeLa cells treated with a TRIP4-overexpression plasmid or hTERT siRNA for 24 h and then exposed to 2, 4, 6, or 8 Gy X-ray irradiation. Survival data was normalized to the unirradiated control cells. The results are expressed as the mean ± SD of at least three independent experiments. *P < 0.05 vs. control. **(H)** HeLa cells were co-transfected with TRIP4-siRNA and hTERT promoter-driven luciferase (−2053/+40) plasmids. Luciferase activity was detected as described before. **(I)** Chromatin immunoprecipitation assays were performed using the hTERT promoter in HeLa cells. **(J)** TRIP4 proteins in the nuclear protein-hTERT probe-streptavidin bead complexes were detected by Western blot using an anti-TRIP4 antibody in HeLa cells. **(K)** A 5′-flanking DNA fragment from position −2053 to +40 (−2053/+40, −1655/+40, −902/+40, −321/+40, −234/+40, −144/+40) of the human hTERT gene was constructed into a promoter-driven luciferase expression vector, pGL3. **(L)** HeLa cells were co-transfected with TRIP4 and different hTERT promoter-driven luciferase plasmids for 48 h. The proteins were extracted, and luciferase activity was detected by a luciferase reporter assay kit. FLAG-lacZ plasmids were used as a negative control. Results are expressed as mean ± SD of at least three independent experiments. *P < 0.05.

factor that drive the transcription of hTERT in cervical cancer, but also indicated that TRIP4 binds to the region of the hTERT promoter at −322 to −902.

3.5. TRIP4 knockdown inhibits cervical cancer progression with downregulation of hTERT expression in a mouse model

To further examine the effects of TRIP4 on tumor growth *in vivo*, we used nude mice bearing cervical cancer xenografts. The HeLa and control cells bearing a stable knockdown of TRIP4 were injected subcutaneously into the underarms of nude mice. As shown in Fig. 5A–E, tumors derived from HeLa cells with the stable knockdown were smaller and lighter in tumor volume and weight than controls. To further verify the effect of TRIP4 knockdown on sensitivity to radiotherapy of cervical cancer cells, we used radiotherapy on nude mice on the seventh day after the injection of tumor cells. We found that the TRIP4-knockdown group was more sensitive than the control group in responding to radiotherapy. In addition, we examined the effects of TRIP4 knockdown on the expression of tumor-associated protein levels in xenografts by Western blotting (Fig. 5F) and IHC analysis (Fig. 5G), and the results showed that TRIP4 knockdown attenuated hTERT, p-AKT, p-ERK, and p-H2AX expression in the xenografts. The above results further demonstrated that TRIP4 plays an important role in the growth of cervical cancer in animals.

3.6. TRIP4 is positively correlated with hTERT expression in clinical tissue samples, and high expression of TRIP4/hTERT predicts a poor prognosis in cervical cancer

To further confirm the role of TRIP4 in the regulation of hTERT, we analyzed their expression relationships in cervical cancer patients with clinical tumors, and we also assessed their relationship with the prognosis of cervical cancer patients. The expression of TRIP4 and hTERT in cervical cancer tissues from 128 cervical cancer patients was detected by IHC assay (Fig. 6A), and 65 cases showed high expression of TRIP4 and hTERT, accounting for 51% of all of the test cases (Table 1). In addition, the relationship between TRIP4 expression and clinical pathology variables was determined and is summarized in Table 2. The expression of TRIP4 was associated with tumor volume and TNM stage. In addition, overall survival (OS) analysis showed that patients with low TRIP4 and hTERT expression had significantly higher 5-OS and a longer survival compared with patients with high expression of both proteins (Fig. 6B and C). These results indicate that TRIP4 is potentially synergistic with hTERT in the prediction of cervical cancer and its expression in patients with cervical cancer.

4. Discussion

In the present study, we demonstrated that TRIP4 promotes cervical cancer growth and radiation resistance after finding high expression of TRIP4 in cervical cancer cell lines and tissue samples. The silencing of TRIP4 inhibited cervical cancer growth both *in vivo* and *in vitro*, and the overexpression of TRIP4 significantly promoted the growth and invasion of cervical cancer cells. We also found that silencing TRIP4 can increase the radiosensitivity of cervical cancer. We further analyzed TRIP4's mechanism of action in cervical cancer, and we demonstrated that it functions in promoting tumor proliferation through the MAPK and PI3K/AKT signaling pathway. We also demonstrated that TRIP4 regulates the expression of hTERT to decrease radiosensitivity in cervical cancer cells. When we explored the relationship between TRIP4 and hTERT expression in tumorous and paracancerous tissues of cervical cancer patients, we found that a high expression of TRIP4 was a potential predictor of poor prognosis. Finally, we showed that silencing TRIP4 inhibited cervical cancer growth and enhanced radiosensitivity in nude mice. We believe this to be the first time that any investigators have revealed the regulation of TRIP4 and its underlying mechanism in cervical cancer.

Some studies have shown that the proliferation and invasion characteristics of cervical cancer are regulated by the MAPK and PI3K/AKT signaling pathway [24–26]. The PI3K/AKT pathway plays a crucial role in the development of cervical cancer, and its downstream elements are promising targets for treatment [27]. We examined the expression of the proteins in this pathway by silencing TRIP4 in cervical cancer cell lines and found that the AKT/MAPK signaling pathway was inhibited. The overexpression of TRIP4 increased the phosphorylation of the pathway-related proteins, suggesting that the promotion of TRIP4 in cervical cancer may also be through the AKT/MAPK signaling pathway. At the same time, the development of cervical cancer was also regulated by hTERT protein, and a previous study has shown that the downregulation of hTERT inhibits the PI3K/AKT signaling pathway in cervical cancer [13]. We found that the knockdown of TRIP4 additionally caused a decrease in hTERT expression, which provided a line of inquiry for our follow-up study.

The main causes of poor prognosis in patients with cervical cancer include tumor cell invasion and metastasis. Previous studies have shown that epithelial-mesenchymal transition (EMT) is an important mechanism that contributes to the metastasis of cervical cancer cells, including MMP upregulation [18] and E-cadherin (tissue inhibitor of metalloproteinases) [19] upregulation caused by metastasis [20]. During metastasis, EMT can reduce adhesion between cancer cells and enhance their ability to metastasize, and MMPs play a key role in the degradation of extracellular matrices of cancer cells that invade other tissues and organs from the primary site. E-cadherin, N-cadherin, MMP-1, and MMP-9 have also been found to be associated with cervical

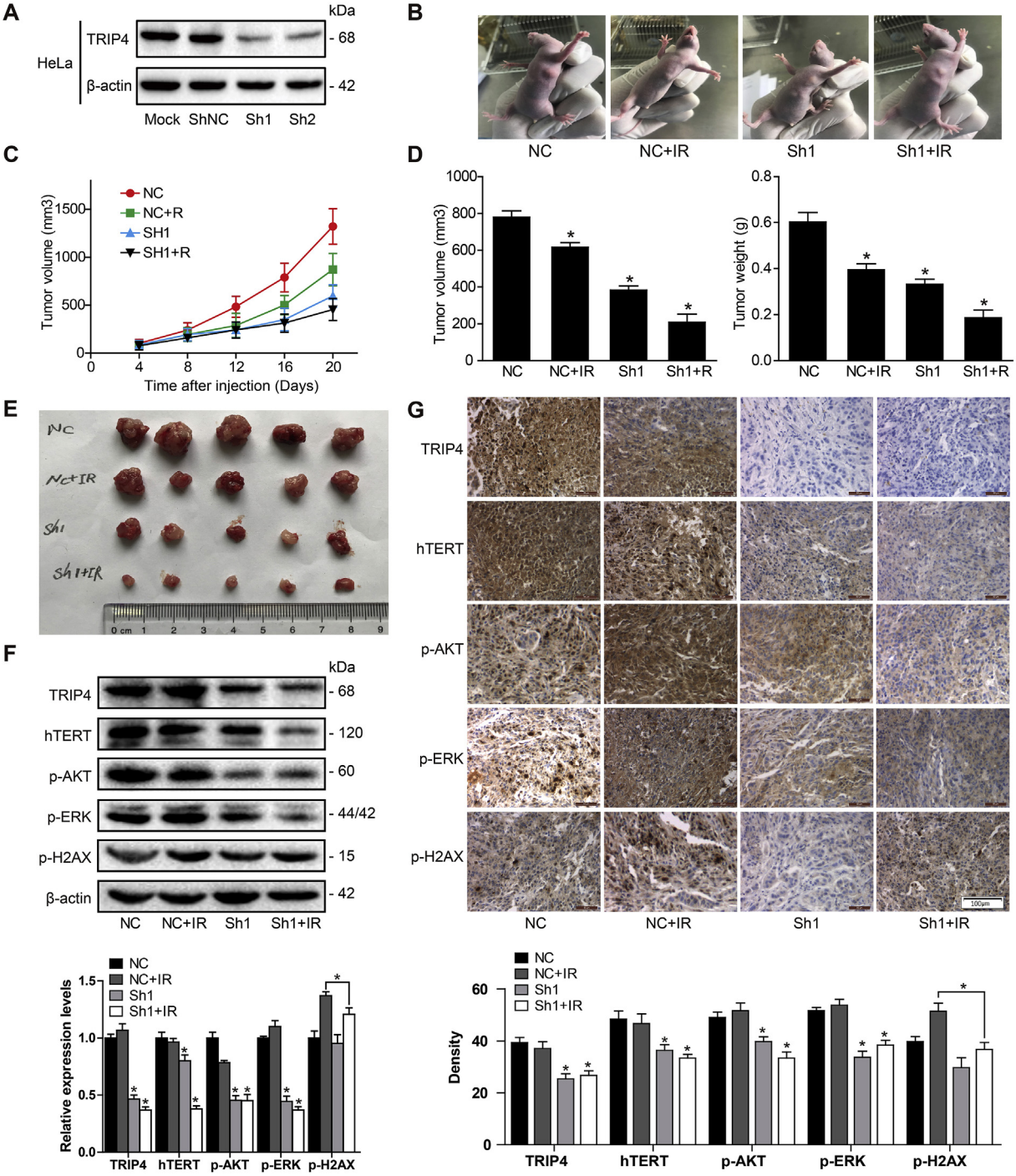


Fig. 5. TRIP4 knockdown inhibited cervical cancer progression concomitant with downregulation of hTERT expression in a mouse model. (A) The expression of TRIP4 in HeLa cells was analyzed by Western blot after transfection with non-specific control shRNA or TRIP4 shRNA. **(B)** Tumors were implanted into female nude mice under the left axilla. **(C)** Dynamic development of tumor volume after cancer cells injection. **(D)** Tumor volume and weight after mice were sacrificed. Data represent the mean ± SD of three independent experiments. *p < 0.05 vs. control. **(E)** The xenografts with HeLa cells were harvested at 21 days after treatment, and pictures of the 4 groups of tumors were obtained. **(F)** The expression levels of TRIP4, hTERT, p-AKT, p-ERK, and p-H2AX proteins in nude mouse tumor tissues were detected by Western blot. **(G)** Immunohistochemistry assays of TRIP4,hTERT,p-AKT,p-ERK and p-H2AX expression from nude mouse tumor tissues in each group. The data represent the mean ± S.D. of three independent experiments. The level of significance is indicated by *P < 0.05.

cancer metastasis [19,21–23]. In our study, we found that silencing TRIP4 regulated the invasion and metastasis of cervical cancer cells, as well as decreased the expression of N-cadherin, MMP-1, MMP-9, and increased the expression of E-cadherin. This indicates that TRIP4 regulates the metastasis of cervical cancer by inhibiting EMT and MMP signaling.

Telomerase is a ribonucleoprotein reverse transcriptase that synthesizes telomere repeats and consists of a RNA template (hTR) and a catalytic protein subunit (hTERT) [28]. hTERT has been reported to be important for cancer tumorigenesis, growth, migration, and invasion [12,29], and it can make cancer cells more resistant to chemotherapeutic agents or radiation therapy via the PI3K/AKT pathway [17]. Cervical cancer patients with high hTERT expression may manifest radiation resistance and produce a subsequently poor prognosis

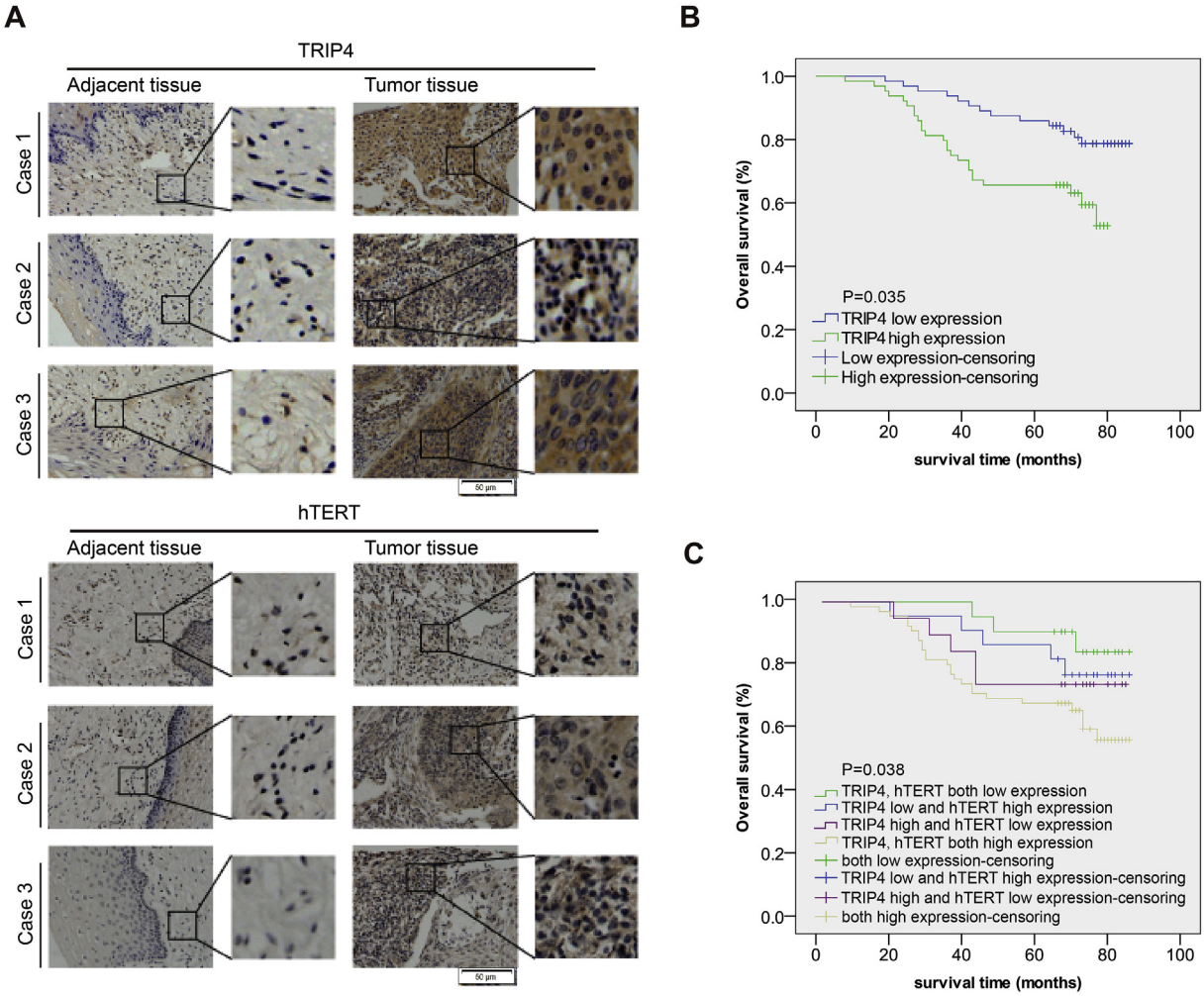


Fig. 6. TRIP4 was positively correlated with hTERT expression in clinical tissue samples. (A) The expression of TRIP4 and hTERT protein from cervical cancer tissue and their corresponding adjacent normal tissues in a microarray was analyzed by immunostaining analysis. **(B)** Kaplan–Meier analysis of overall survival for cervical cancer patients with different expression levels of TRIP4 by log-rank test. **(C)** Kaplan–Meier analysis of overall survival for cervical cancer patients with different expression levels of TRIP4 and hTERT by log-rank test.

Table 1
Cox-regression analyses for the prognosis of 128 cervical cancer patients.

Clinical prognostic factors (n = 128)			
Clinical factor	RR	95% CI	P value
Metastasis (N0 + N1)/(N2 + N3)	1.177	0.562–2.466	0.665
TNM (I + II)/(III + IV)	2.396	1.049–5.476	0.038*
TRIP4	1.611	0.786–3.302	0.193

Abbrevations: RR, relative risk; CI, confidence interval, *P < 0.05.
The expression of TRIP4 and hTERT in cervical tumor tissues from 128 cervical cancer. Notes: Clinical stage TNM (I + II)/(III + IV) and TRIP4 expression were also associated with survival of the patients with cervical cancer based on Univariate analysis of Cox regression model. * (P < 0.05).

[30,31]. In our study, the expression of TRIP4 in cervical cancer was associated with the expression of hTERT both *in vivo* and *in vitro*.

Radiation therapy is one of the principal effective treatments for cervical cancer [1,2]. Phosphorylation of histone H2AX occurs in response to DNA double-strand breaks (DSB) produced by ionizing radiation and a variety of genotoxic drugs [32]; this response constitutes an early marker of a cell's response to DNA damage, particularly if the damage involves the formation of DSBs [33]. RAD51 is one of the pivotal enzymes for DNA DSB repair by the homologous recombination (HR) pathway, which suggests its use as a promising and novel target for cervical cancer [34]. We found that after radiotherapy exposure to cervical cancer cells after TRIP4 knockdown, the expression of the above DNA damage repair protein was decreased. Recent studies have reported that the inhibition of hTERT may exert radiosensitizing effects on cervical cancer [35]. Therefore, we hypothesized that TRIP4 was involved in the regulation of hTERT expression, which in turn would affect proliferation inhibition and radiosensitization of cervical cancer. To test this hypothesis, we performed a luciferase experiment that initially proved that TRIP4 was involved in the regulation of the promoter region of hTERT. We then used a streptavidin–biotin pulldown assay and chromatin immunoprecipitation analysis to find evidence that TRIP4 was involved in the regulation of hTERT expression.

In our study, we also performed IHC on tissue microarrays using tumorous tissues and paracancerous tissues from 128 cervical cancer patients, and we analyzed the expression of TRIP4 and hTERT. We found that the expression of TRIP4 and hTERT increased significantly in tumorous tissues, which has also been observed in other recent reports [36]. Our statistical analysis additionally found that patients with an increased expression of TRIP4 and hTERT had poor prognosis. Finally, we implanted TRIP4-silenced HeLa cells into nude mice, and we treated the experimental group with radiation therapy. After extracting the tumors, we performed IHC and Western immunoblotting to verify the aforementioned results.

In conclusion, our study showed that TRIP4 promotes cervical

Table 2
Correlation analyses of TRIP4 protein expression in relation to clinicopathologic variables of 128 cervical cancer patients.

Clinicopathologic characteristic (n = 128)			
Clinical factor	TRIP4 expression		P value
	High (%)	Low (%)	
Age			0.038*
≤60	52 (78.8)	14	
> 60	33 (56.9)	25	
T			0.014*
T1/T2	61 (61)	39	
T3/T4	24 (85.7)	4	
N			0.123
N0/N1	64 (63.4)	37	
N2/N3	21 (77.8)	6	
M			0.475
M0	0 (0)	0	
M1	1 (100)	0	
TNM			0.007*
I/II	58 (58.6)	41	
III/IV	27 (93.1)	2	
h-TERT (%)			0.004*
High	[a]65 (74.7)	22	
Low	20 (48.8)	21	

*P < 0.05.
The relationship between TRIP4 expression and clinical pathology. Notes: [a]65 cases showed high expression of TRIP4 and hTERT, accounting for 51% of all of the test cases and high expression of TRIP4 was associated with Age, Tumor size, TNM stages and hTERT expression *(P < 0.05) according to Pearson chi-square test.

cancer growth and by targeting the MAPK and PI3K/AKT pathway. In addition, we found that TRIP4 up-regulates hTERT expression by binding to the promoter region of the hTERT gene in cervical cancer cells which affected radiosensitivity. We therefore suggest the development of TRIP4 as a novel anticancer target in cervical cancer therapy.

Author contributions

LZ, WD, YC, YL, and KZ conceived the study, participated in the designing of the study, interpreted, drafted the manuscript, and coordinated the study. YC, YL, FZ, KZ, ZL, MC, SH, CT, WY and WG performed the experiments and analyzed and prepared data for publication. YC, KZ, ZL, and ML performed cell culture, western blotting, flow cytometry, immunohistochemistry, animal experiments and statistical analyses. YC, ZL, and KZ performed dual luciferase assays and co-immunoprecipitation assays. YC, YL, FZ, and ML performed immunofluorescence and Transwell invasion assays.

Disclosure of potential conflicts of interest

No potential conflicts of interest are disclosed.

Acknowledgements

This work was supported by the funds from the National Natural Science Foundation of China (81473452 LZ, 81703904 KZ, 81472178 WD, 81272195 WD), the Education Department of Liaoning Province, China ("the Program for Distinguished Professor of Liaoning Province", "the Scientific Research Project, L2015142", and Construction Project of Traditional Chinese Medicine of Liaoning Province).

References

[1] G.P. Yee, P. de Souza, L.M. Khachigian, Current and potential treatments for cervical cancer, Curr. Cancer Drug Targets 13 (2013) 205–220.
[2] J. Fang, H. Zhang, S. Jin, Epigenetics and cervical cancer: from pathogenesis to therapy, Tumour Biol. 35 (2014) 5083–5093.
[3] A.C. de Freitas, C. Gomes Leitao Mda, E.C. Coimbra, Prospects of molecularly-targeted therapies for cervical cancer treatment, Curr. Drug Targets 16 (2015) 77–91.
[4] S.W. Kim, H.J. Kim, D.J. Jung, S.K. Lee, Y.S. Kim, J.H. Kim, T.S. Kim, J.W. Lee, Retinoid-dependent antagonism of serum response factor transactivation mediated by transcriptional coactivator proteins, Oncogene 20 (2001) 6638–6642.
[5] J.W. Lee, H.S. Choi, J. Gyuris, R. Brent, D.D. Moore, Two classes of proteins dependent on either the presence or absence of thyroid hormone for interaction with the thyroid hormone receptor, Mol. Endocrinol. 9 (1995) 243–254.
[6] D.J. Jung, H.S. Sung, Y.W. Goo, H.M. Lee, O.K. Park, S.Y. Jung, J. Lim, H.J. Kim, S.K. Lee, T.S. Kim, J.W. Lee, Y.C. Lee, Novel transcription coactivator complex containing activating signal cointegrator 1, Mol. Cell Biol. 22 (2002) 5203–5211.
[7] S. Almeida-Vega, K. Catlow, S. Kenny, R. Dimaline, A. Varro, Gastrin activates paracrine networks leading to induction of PAI-2 via MAZ and ASC-1, Am. J. Physiol. Gastrointest. Liver Physiol. 296 (2009) G414–G423.
[8] J. Hao, H. Xu, M. Luo, W. Yu, M. Chen, Y. Liao, C. Zhang, X. Zhao, W. Jiang, S. Hou, X. Feng, K. Zou, Y. Chen, W. Huang, W. Guo, L. Kang, W. Deng, The tumor-promoting role of TRIP4 in melanoma progression and its involvement in response to BRAF-targeted therapy, J. Investig. Dermatol. 138 (2018) 159–170.
[9] Y.S. Cong, W.E. Wright, J.W. Shay, Human telomerase and its regulation, Microbiol. Mol. Biol. Rev. 66 (2002) 407–425 (table of contents).
[10] S.Y. Ying, J.X. Xiong, H.X. Mai, J.J. Lin, L.N. Jiang, L. Cheng, Q. Ye, Advances on the regulation of telomerase, Yi Chuan 38 (2016) 289–299.
[11] M.A. Blasco, Telomeres and cancer: a tale with many endings, Curr. Opin. Genet. Dev. 13 (2003) 70–76.
[12] C. Cifuentes-Rojas, D.E. Shippen, Telomerase regulation, Mutat. Res. 730 (2012) 20–27.
[13] Y.A. Shi, Q. Zhao, L.H. Zhang, W. Du, X.Y. Wang, X. He, S. Wu, Y.L. Li, Knockdown of hTERT by siRNA inhibits cervical cancer cell growth in vitro and in vivo, Int. J. Oncol. 45 (2014) 1216–1224.
[14] K. Kurvinen, V. Rantanen, S. Syrjanen, B. Johansson, Radiation-induced effects on telomerase in gynecological cancer cell lines with different radiosensitivity and repair capacity, Int. J. Radiat. Biol. 82 (2006) 859–867.
[15] N. Serakinci, R. Christensen, J. Graakjaer, C.J. Cairney, W.N. Keith, J. Alsner, G. Saretzki, S. Kolvraa, Ectopically hTERT expressing adult human mesenchymal stem cells are less radiosensitive than their telomerase negative counterpart, Exp. Cell Res. 313 (2007) 1056–1067.
[16] F.A. Goytisolo, E. Samper, J. Martin-Caballero, P. Finnon, E. Herrera, J.M. Flores, S.D. Bouffler, M.A. Blasco, Short telomeres result in organismal hypersensitivity to ionizing radiation in mammals, J. Exp. Med. 192 (2000) 1625–1636.
[17] R. Ram, O. Uziel, O. Eldan, E. Fenig, E. Beery, S. Lichtenberg, Y. Nordenberg, M. Lahav, Ionizing radiation up-regulates telomerase activity in cancer cell lines by post-translational mechanism via ras/phosphatidylinositol 3-kinase/Akt pathway, Clin. Cancer Res. 15 (2009) 914–923.
[18] Y. Wu, T.T. Gu, P.S. Zheng, CIP2A cooperates with H-Ras to promote epithelial-mesenchymal transition in cervical-cancer progression, Cancer Lett. 356 (2015) 646–655.
[19] J.W. Miao, L.J. Liu, J. Huang, Interleukin-6-induced epithelial-mesenchymal transition through signal transducer and activator of transcription 3 in human cervical carcinoma, Int. J. Oncol. 45 (2014) 165–176.
[20] H. Liu, J. Xiao, Y. Yang, Y. Liu, R. Ma, Y. Li, F. Deng, Y. Zhang, COX-2 expression is correlated with VEGF-C, lymphangiogenesis and lymph node metastasis in human cervical cancer, Microvasc. Res. 82 (2011) 131–140.
[21] Y. Nishioka, S. Sagae, A. Nishikawa, S. Ishioka, R. Kudo, A relationship between Matrix metalloproteinase-1 (MMP-1) promoter polymorphism and cervical cancer progression, Cancer Lett. 200 (2003) 49–55.
[22] C. Song, S. Zhu, C. Wu, J. Kang, Histone deacetylase (HDAC) 10 suppresses cervical cancer metastasis through inhibition of matrix metalloproteinase (MMP) 2 and 9 expression, J. Biol. Chem. 288 (2013) 28021–28033.
[23] M. Feng, Y. Wang, K. Chen, Z. Bian, W. Jinfang, Q. Gao, IL-17A promotes the migration and invasiveness of cervical cancer cells by coordinately activating MMPs expression via the p38/NF-kappaB signal pathway, PLoS One 9 (2014) e108502.
[24] E. Jiang, X. Sun, H. Kang, L. Sun, W. An, Y. Yao, X. Hu, Dehydrocostus lactone inhibits proliferation, antiapoptosis, and invasion of cervical cancer cells through PI3K/akt signaling pathway, Int. J. Gynecol. Cancer 25 (2015) 1179–1186.
[25] K.P. Srinivas, R. Viji, V.M. Dan, I.S. Sajitha, R. Prakash, P.V. Rahul, T.R. Santhoshkumar, S. Lakshmi, M.R. Pillai, DEPTOR promotes survival of cervical squamous cell carcinoma cells and its silencing induces apoptosis through down-regulating PI3K/AKT and by up-regulating p38 MAP kinase, Oncotarget 7 (2016) 24154–24171.
[26] J.L. Perfettini, M. Castedo, R. Nardacci, F. Ciccosanti, P. Boya, T. Roumier, N. Larochette, M. Piacentini, G. Kroemer, Essential role of p53 phosphorylation by p38 MAPK in apoptosis induction by the HIV-1 envelope, J. Exp. Med. 201 (2005) 279–289.
[27] J. Wu, C. Chen, K.N. Zhao, Phosphatidylinositol 3-kinase signaling as a therapeutic target for cervical cancer, Curr. Cancer Drug Targets 13 (2013) 143–156.
[28] M.J. McEachern, A. Krauskopf, E.H. Blackburn, Telomeres and their control, Annu. Rev. Genet. 34 (2000) 331–358.
[29] J.F. Noel, R.J. Wellinger, Exposing secrets of telomere-telomerase encounters, Cell 150 (2012) 453–454.
[30] P. Moreno-Acosta, A. Vallard, S. Carrillo, O. Gamboa, A. Romero-Rojas, M. Molano, J. Acosta, D. Mayorga, C. Rancoule, M.A. Garcia, M. Cotes Mestre, N. Magne, Biomarkers of resistance to radiation therapy: a prospective study in cervical carcinoma, Radiat. Oncol. 12 (2017) 120.
[31] H. Yang, H. Zhang, Y. Zhong, Q. Wang, L. Yang, H. Kang, X. Gao, H. Yu, C. Xie, F. Zhou, Y. Zhou, Concomitant under expression of TGFBR2 and overexpression of

hTERT are associated with poor prognosis in cervical cancer, Sci. Rep. 7 (2017) 41670.
[32] T.T. Paull, E.P. Rogakou, V. Yamazaki, C.U. Kirchgessner, M. Gellert, W.M. Bonner, A critical role for histone H2AX in recruitment of repair factors to nuclear foci after DNA damage, Curr. Biol. 10 (2000) 886–895.
[33] B. Meyer, K.O. Voss, F. Tobias, B. Jakob, M. Durante, G. Taucher-Scholz, Clustered DNA damage induces pan-nuclear H2AX phosphorylation mediated by ATM and DNA-PK, Nucleic Acids Res. 41 (2013) 6109–6118.
[34] Q. Chen, D. Cai, M. Li, X. Wu, The homologous recombination protein RAD51 is a promising therapeutic target for cervical carcinoma, Oncol. Rep. 38 (2017) 767–774.
[35] R. Wang, F. Lin, X. Wang, P. Gao, K. Dong, S.H. Wei, S.Y. Cheng, H.Z. Zhang, The therapeutic potential of survivin promoter-driven siRNA on suppressing tumor growth and enhancing radiosensitivity of human cervical carcinoma cells via downregulating hTERT gene expression, Cancer Biol. Ther. 6 (2007) 1295–1301.
[36] H.Y. Wang, G. Kim, H. Cho, S. Kim, D. Lee, S. Park, K.H. Park, H. Lee, Diagnostic performance of HPV E6/E7, hTERT, and Ki67 mRNA RT-qPCR assays on formalin-fixed paraffin-embedded cervical tissue specimens from women with cervical cancer, Exp. Mol. Pathol. 98 (2015) 510–516.