

Knockdown of *Trio* by CRISPR/Cas9 suppresses migration and invasion of cervical cancer cells

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Abstract. Triple functional domain protein (Trio) is an evolutionarily conserved protein with guanine nucleotide exchange factors that regulate different physiological processes in some types of cancer. However, the expression and function of Trio in cervical cancer are still unknown. The purpose of this study was to detect the expression of Trio in cervical cancer tissues and to evaluate its clinical value. Furthermore, the effects of the Trio on the migration and invasion of cervical cancer cells and its mechanism were investigated *in vitro*. The results of the present study revealed that *Trio* expression levels were significantly higher in most of the clinical cervical cancer samples than in adjacent tissues. The clinicopathological significance of *Trio* expression was also analyzed, and the results revealed that high expression levels in cervical cancer were correlated with lymph node metastasis ($P=0.005$). The CRISPR/Cas9 system was used to knockdown the endogenous Trio. The inhibition of Trio significantly decreased the migration and invasion abilities of cervical cancer cells. Meanwhile, levels of RhoA/ROCK signaling factors (RhoA, Rock, and p-LIMK), which contributed to cell migration and invasion, were decreased along with the inhibition of Trio. Therefore, Trio may regulate the migration and invasion of cervical cancer through the RhoA/ROCK signaling pathway.

Introduction

Triple functional domain protein (Trio) is a large evolutionarily conserved protein that harbors a serine/threonine kinase domain and two guanine nucleotide exchange factor

(GEF) domains (1). The two GEF domains activate the GTPases Rac1/RhoG and RhoA, potentially linking several Rho-GTPase signaling pathways (2). Trio participates in the regulation of different physiological processes, most notably in neuronal physiology (3-7). In addition to its main effects on neuronal development, Trio plays important roles in other aspects of myogenesis and phagocytosis (8-10). Since Trio controls a wide range of cellular processes, dysregulation of its activity is related to the emergence of diseases. Trio was demonstrated to be highly upregulated in urinary bladder tumors (11), soft tissue sarcomas (12), breast cancer (13), and various other types of cancer (11-19). Higher Trio levels were closely related to invasive tumor phenotype, high tumor grade, and rapid tumor cell proliferation in breast tumors (13). A high expression level of Trio was also associated with clinicopathological parameters and poor prognosis of hepatocellular carcinoma patients. Trio was also expressed at higher levels in glioblastoma compared with low-grade glioma and was related to poor patient survival (17). Concomitantly, Trio was capable of serving as a prognostic marker, which helped in determining the therapeutic modality of colorectal cancer patients (19).

Cervical carcinoma is the second most common cause of cancer mortality in females and seriously affects the health of women worldwide (20). Tumor metastasis results in ~90% of cancer-related deaths (21,22). For cervical cancer, invasion and migration are important processes in the progression of cancer metastasis (23). Therefore, studies on molecular mechanisms underlying tumor invasion and metastasis are very relevant for understanding tumor occurrence and development. Despite the major advances in therapeutic approaches, most patients still succumb to cancer progression (24-27). Lymph node metastasis acts as an important unfavorable prognostic factor and a primary cause of cancer-related deaths (28). Previous studies have reported that Trio was involved in some types of cancer development (11-19). High expression of Trio was associated with rapid tumor cell proliferation and invasive tumor growth (18). However, studies on the role of Trio and its clinical significance in cervical cancer remain non-existent. In the present study, we identified Trio expression in cervical cancer and its effects on cell invasion and migration. Furthermore, we investigated the mechanism of Trio in cervical cancer migration and invasion.

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Table I. The sequences and location of gRNAs targeting *Trio*.

Name	Genomic target	Target location
g-RNA1	TCCGCCGCTGCTGCCGCTCATGG	Exon 1
g-RNA2	GCCGCGCTGGCCGCCGCGGCGGG	Exon 1
g-RNA3	CACCTCTGAGCCAGGGATACTGG	Exon 24

Materials and methods

Tissue specimens and cell lines. All procedures adhered to the approved medical ethics practices and the Human Ethics Review Board of the Second Hospital of Shandong University, China [Approval no.: KYLL-2014 (LW) P-001]. Clinical specimens were collected from patients at the Second Hospital of Shandong University from October 2014 to December 2016. Histological classifications and clinical staging were based on the classification system by the International Federation of Gynecology and Obstetrics (International Federation of Gynecology and Obstetrics Cancer Committee; FIGO, 2009) (29). A tumor size of 4 cm was chosen as a 'watershed' value according to previous studies (30-32). The human cervical cancer cell lines, Caski and HeLa, and normal cervical cell, CRL-2614, were purchased from the Cell Bank of the Chinese Academy of Sciences (Shanghai, China). The cells were grown in RPMI-1640 medium supplemented with 10% fetal bovine serum (FBS) at 37°C and 5% CO₂. Transfection was performed with Lipofectamine 2000 reagent (Invitrogen; Thermo Fisher Scientific, Waltham, MA, USA) following the manufacturer's protocols.

Reverse transcription-quantitative polymerase chain reaction analysis. Total RNA was isolated from tissue and cells using TRIzol reagent (Invitrogen), according to manufacturer's protocols. Total RNA (1 µg) was reverse-transcribed into cDNA using PrimeScript RT reagent kit (Takara Biotechnology Co., Ltd., Dalian, China), and qPCR was conducted using SYBR-Green dye mix (Invitrogen). Thermocycling conditions for RT-qPCR were as follows: 95°C for 10 min, 40 cycles of 95°C for 20 sec, 55°C for 30 sec, and 72°C for 20 sec. The relative expression level of *Trio* was calculated using 2^{-ΔΔC_q}, and the expression level of *Trio* mRNA was normalized to that of GAPDH. The *Trio* sense and antisense primers were designed as follows: 5'-AGATTCTCTGTCGTTAAG-3', and 5'-TTCTAAGACCAGGATGTA-3', respectively. The GAPDH sense and antisense primers were: 5'-CTCAAGATCATCAGCAAT-3' and 5'-CGATACCAAAGTTGTCAT-3', respectively.

CRISPR/Cas9 system targeting human *Trio*. The guide RNA sequences targeting the human *Trio* gene were designed using an online sgRNA design tool at <https://crispr.mit.edu/>. Guide sequences with high scores for on-target activity and minimal predicted off-target activity were selected. Table I displays the three sets of oligonucleotides. Primer oligonucleotides were annealed under 88°C for 2 min, 65°C for 10 min, 37°C for 10 min, and 25°C for 5 min. The annealing primer was then purified and cloned into the PX330 vector (Addgene,

Cambridge, MA, USA). The CRISPR/Cas9 backbone and CRISPR/Cas9-gRNA plasmids were separately transfected into cells using a liposome.

In vitro invasion assay. For the invasion assay, a Transwell chamber placed into a 24-well plate was coated with 30 µl of Matrigel and incubated for 40 min at 37°C. In the Transwell assay, the cells were trypsinized and seeded in chambers at a density of 5x10⁵ cells/well and were cultured in serum-free medium, while 500 µl of 10% FBS-RPMI-1640 were added to the lower chamber. After 24 h, the cells on the upper surface were removed, and the migrated cells were fixed with 100% methanol for 30 min. The cells on the bottom surface of the membrane were stained with eosin for 20 min. The cell images were obtained under a phase-contrast microscope.

Wound healing assays. Cells were seeded in 6-well plates. When the cells reached 90-100% confluence, the cell monolayers were wounded by scraping with a micropipette tip. The spreading of wound closure was observed after 48 h. Images were captured using a phase-contrast microscope (Olympus, Tokyo, Japan) either immediately or 48 h after wounding. All experiments were repeated thrice.

Western blot analysis. Western blot analysis was performed using anti-Trio (Rabbit, Polyclonal, 1:1,000; Abnova, Taipei, Taiwan), anti-RhoA (Rabbit, monoclonal, 1:1,000, cat. no. 2117; Cell Signaling Technology, Beverly, MA, USA), anti-Rock1 (Mouse, monoclonal, 1:800, cat. no. sc-365628), anti-Rock2 (Mouse, monoclonal, 1:800, cat. no. sc-398519) (both from Santa Cruz Biotechnology, Santa Cruz, CA, USA), and anti-LIMK1 (Rabbit, Polyclonal, 1:1,000, cat. no. 3842) antibodies, p-LIMK1 (Thr508) (Rabbit, Polyclonal, 1:1,000, cat. no. 3841) (both from Cell Signaling Technology), with rabbit anti-β-actin (Mouse, monoclonal, 1:5,000; Bioworld, Nanjing, China) were used as a loading control. Band intensities of the western blotting were analyzed using Image Analysis Software v2.0 (Thermo Fisher Scientific Inc.).

RhoA activation assay. RhoA activity was assessed using a Rho Activation Assay Biochem kit™ (Cytoskeleton, Inc., Denver, CO, USA) according to the manufacturer's protocol.

Statistical analysis. All values were expressed as the mean ± SD of three individual experiments. Statistical analyses were performed using Student's t-test. When appropriate, the Mann-Whitney U-test was used to compare the two groups. P<0.05 were considered to indicate a statistically significant result. SPSS software was used for statistical analyses.

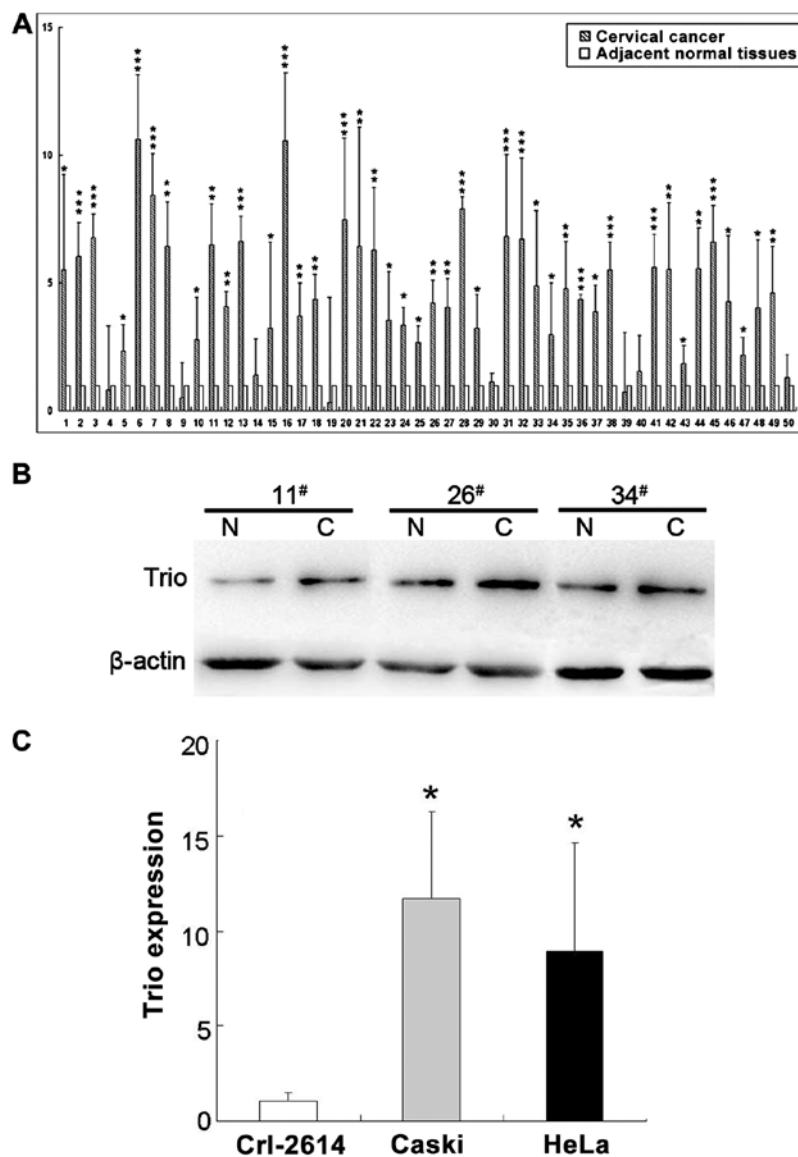


Figure 1. Expression of *Trio* in cervical cancer tissues and cell lines. (A) Relative mRNA expression of *Trio* was detected by qRT-PCR in 50 paired human cervical cancer tissues. (B) *Trio* protein levels in three paired randomly selected cervical cancer tissues and their corresponding adjacent normal tissues were determined using western blot assays. (C) Expression of *Trio* in normal cervical cell line Crl-2614 and cervical cancer cell lines HeLa and Caski. * $P<0.05$; ** $P<0.01$; *** $P<0.001$.

Results

Expression of *Trio* in cervical cancer biopsies and cell lines.

To ascertain *Trio* expression in cervical cancer, we performed real-time PCR on 50 clinical specimens from cervical cancer patients. The expression levels of *Trio* in the tumor tissues for all 50 samples were significantly higher than that in the matching adjacent tissue (Fig. 1A). Furthermore, we determined the protein level of *Trio* in randomly selected cancer and adjacent normal tissues ($n=3$). The protein level was also increased compared to the normal tissues (Fig. 1B). The expression of *Trio* was also determined in cervical cancer cell lines (Caski and HeLa) and in a normal cervical cell line (Crl-2614) (Fig. 1C), where the results obtained from the two analyses were in agreement with the data obtained from real-time PCR, indicating the significant increase of *Trio* levels in cervical cancer tissues and cell lines compared with normal cervical tissue and a cell line.

High expression of Trio in cervical cancer is correlated with tumor metastasis. We analyzed the expression level of *Trio* to determine its clinicopathological significance. Notably, *Trio* levels were significantly increased in patients with lymph node involvement ($P=0.005$) (Table II). Therefore, *Trio* expression associates with lymph node metastasis and is a potential diagnostic marker for cervical cancer.

Inhibition of Trio expression by CRISPR/Cas9. We used Caski cells to examine the effects of *Trio* gene knockdown by CRISPR/Cas9. We transfected Caski cells with the control, CRISPR+Cas9+gRNA empty, CRISPR+Cas9+*Trio*-1, CRISPR+Cas9+*Trio*-2, and CRISPR+Cas9+*Trio*-3 and cultured them for two days. Fig. 2A revealed the transfection results as determined by western blotting, which confirmed the reduced expression of *Trio* by CRISPR+Cas9-gRNA1 in comparison with the control at 48 h. CRISPR+Cas9+*Trio*-1 exhibited effective knockdown of *Trio* gene expression at

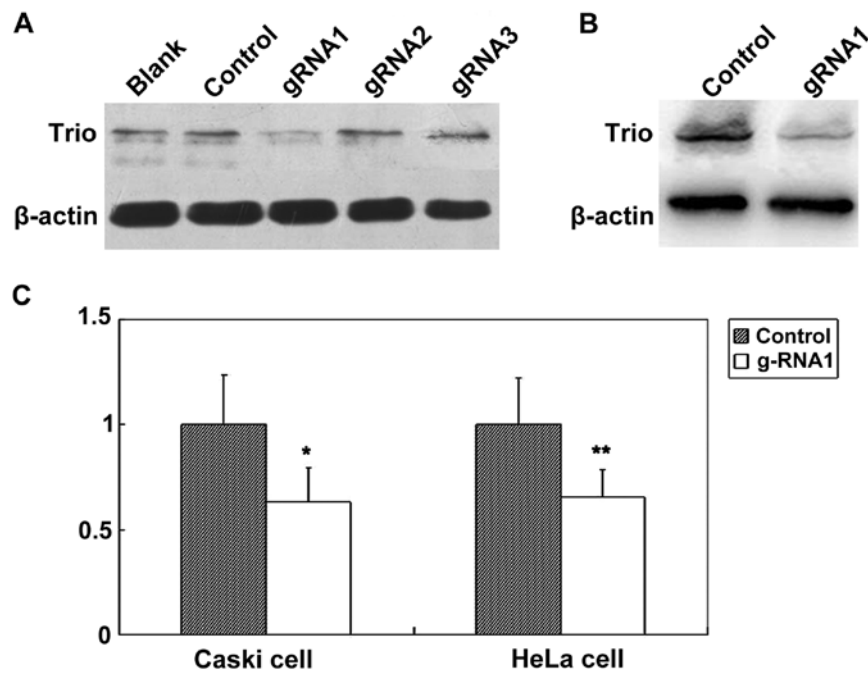


Figure 2. Knockdown of *Trio* expression by transfection of CRISPR/Cas9. (A) CRISPR+Cas9+*Trio*-1 exhibited effective knockdown of *Trio* gene expression in Caski cells. (B) CRISPR+Cas9+*Trio*-1 exhibited effective knockdown of *Trio* gene expression in HeLa cells. (C) The data are presented as the mean \pm SD of three independent experiments. Control, gRNA empty vector. * $P < 0.05$; ** $P < 0.01$.

Table II. The association between *Trio* expression with clinicopathological parameters in 50 cervical cancer patients.

Variables	No.	<i>Trio</i> relative transcript level (mean mRNA/GAPDH \pm standard deviation)	P-value
Age (years)			0.156 ^a
≤50	18	0.0336 \pm 0.0309	
>50	32	0.0360 \pm 0.0239	
Tumor size (cm)			0.856 ^a
≤4	37	0.0340 \pm 0.0249	
>4	13	0.0368 \pm 0.0376	
FIGO staging			0.983 ^b
I	31	0.0363 \pm 0.0330	
II	10	0.0351 \pm 0.0235	
III-IV	9	0.0361 \pm 0.0257	
Histological grade			0.996 ^b
Well	13	0.0365 \pm 0.0351	
Moderately	10	0.0365 \pm 0.0321	
Poorly	27	0.0370 \pm 0.0300	
Pelvic lymph node metastasis			0.005 ^a
No	34	0.0341 \pm 0.0348	
Yes	16	0.0423 \pm 0.0299	

^aComparisons between groups using the Mann-Whitney test. ^bComparisons among three groups performed by one-way analysis of variance (ANOVA). FIGO, International Federation of Gynecology and Obstetrics Cancer Committee.

48 h (Fig. 2A and C). Significantly decreased *Trio* expression was also demonstrated in the HeLa cell line (Fig. 2B and C) as determined using western blotting. Therefore, we obtained an effective gRNA for *Trio*.

Knockdown of *Trio* by CRISPR/Cas9 inhibits cervical cancer cell migration and invasion. Migration assay through wound-healing revealed that the migration ability of Caski and HeLa cells transfected with the control was significantly higher in cervical cancer cells than that of cells transfected with knocked down *Trio*-1 (Fig. 3).

Invasion assay using the Transwell method revealed that the invasiveness of Caski and HeLa cells transfected with CRISPR+Cas9+*Trio*-1 was significantly lower than that of cells transfected with the control (Fig. 4). *Trio* silencing inhibited cervical cancer cell migration and invasion via inactivation of the RhoA/Rock signaling pathway.

Rho-GTPase proteins participate in cell motility and actin cytoskeleton reorganization (18). RhoA is a member of the Rho-GTPase proteins and acts as a switch between the active GTP-bound form and inactive GDP-bound form. The activated RhoA-GTPase is associated with invasive cancers and tumor metastasis (30,31). To determine whether the inactivation of the Rho-GTPase pathway mediated the inhibitory effect of *Trio* on cervical cancer cell migration and invasion, we examined the expression level of the active RhoA-GTPase under *Trio* knockdown by western blotting. The active form of GTP-bound RhoA was downregulated after *Trio* decreased. We assessed the protein levels of ROCK1, ROCK2 and p-LIMK1, a protein directly downstream of ROCK, after transient transfection of CRISPR+Cas9+gRNA empty and CRISPR+Cas9+*Trio*-1 for 48 h. Knockdown of *Trio* significantly inhibited the protein expression of activated RhoA, ROCK1, ROCK2 and p-LIMK1 compared with the control (Fig. 5), indicating that *Trio* may

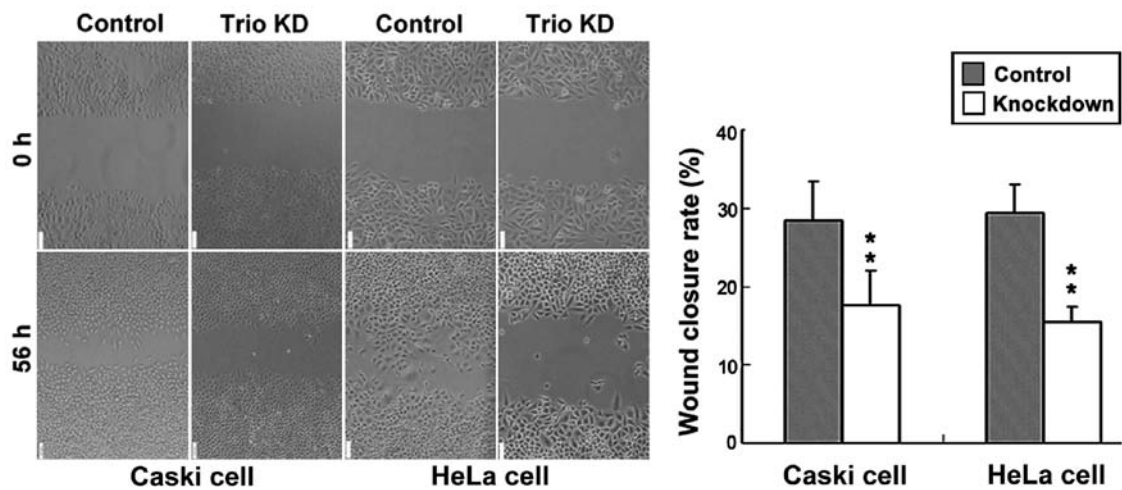


Figure 3. A scratch wound-healing assay was conducted in Caski and HeLa cells transfected with CRISPR+Cas9+*Trio-1* (*Trio* knockdown) or CRISPR+Cas9+gRNA empty (control). The migration distance was assessed at 0 and 56 h following cell-scratching. Images are viewed under a magnification of x100. **P<0.05.

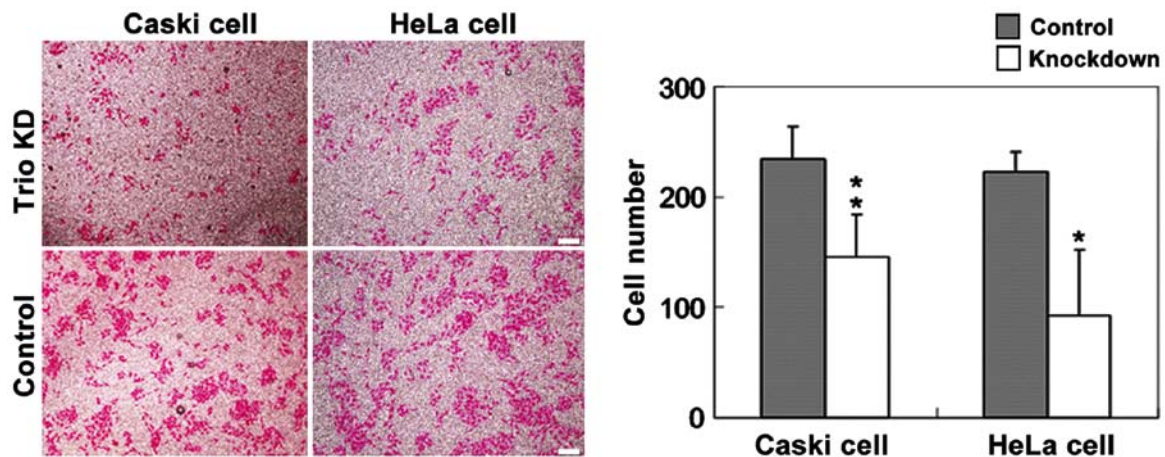


Figure 4. A Transwell assay revealed the invasion capacity of Caski and HeLa cells transfected with CRISPR+Cas9+*Trio-1* (*Trio* knockdown) or CRISPR+Cas9+gRNA empty (control). The cells were stained with 1% eosin. Images were viewed under a magnification of x100. The average number of invading cells is from three independent experiments. *P<0.05; **P<0.01.

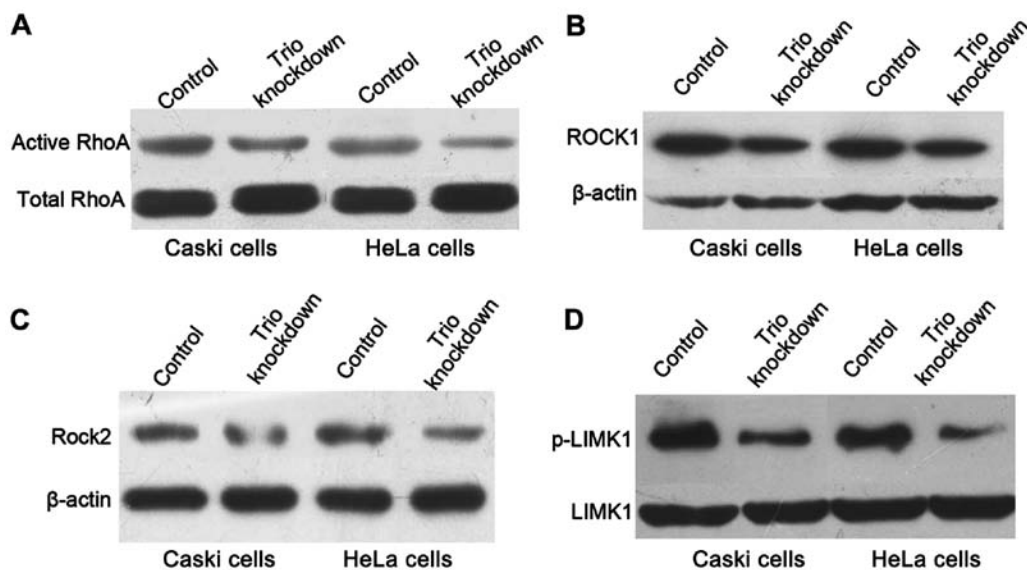


Figure 5. The expression levels of active RhoA, ROCK1, ROCK2, and p-LIMK1 were detected using western blotting in Caski and HeLa cells transfected with CRISPR+Cas9+*Trio-1* or the control for 48 h.

mediate the metastatic ability of cervical cancer cells through inactivation of the RhoA-GTPase pathway.

Discussion

In the present study, we first determined the mRNA expression levels of *Trio* in 50 pairs of human cervical cancer tissues and the matching adjacent tissues by qRT-PCR. Our data indicated that *Trio* expression was higher in cervical cancer tissues than that in adjacent tissues. Consistent with the results of tissue analysis, the expression of *Trio* at the RNA level was also increased in cervical cancer cell lines compared with the normal cervical cells. Furthermore, the clinicopathological parameters were evaluated to identify the correlation between *Trio* expression and clinical characteristics. The elevated expression of *Trio* was significantly associated with lymph node metastasis in cervical cancer patients. Our results revealed that the high expression of *Trio* was related to lymph node metastasis and serves as a potential diagnostic marker for cervical cancer.

Cell migration and invasion are key features for metastatic dissemination of cancer cells and metastatic formation, which are the leading causes of death in cancer patients (33). In the present study, we performed scratch wound and Transwell invasion assays to examine the effect of *Trio* expression on cervical carcinoma cell migration and invasion, respectively, and our results revealed that *Trio* knockdown significantly inhibited cervical cancer cell migration and invasion.

Most cancer cells control their migratory and invasive capabilities by actin cytoskeleton reorganization (34). Rho-family small GTPases, which are activated by guanine nucleotide exchange factors (GEFs), are key regulators of cytoskeleton dynamics (35,36). *Trio* is a member of the RhoGEFs family, and has two GEF domains, one for RAC (GEF1) and the other (GEF2) for RHO (1,2). The Rho/ROCK signaling pathway participates in tumor growth and metastasis by regulating actin cytoskeleton reorganization (37,38). Western blotting demonstrated that *Trio* knockdown downregulated the activity of RhoA in Caski and HeLa cells. Previous studies have demonstrated that ROCK kinase, activated by RhoA, is a key regulator of intracellular signaling pathways that contributes to cell migration and invasion. A high expression of ROCK induces migration and invasion in several types of tumor (39-42). In our study, the protein level of ROCK1 and ROCK2 were also decreased during *Trio* knockdown. We also examined the phosphorylation of LIM-kinase 1 (LIMK1), which is activated by the small GTPase Rho and its downstream protein kinase ROCK (42) and is important for the regulation of actin cytoskeletal reorganization. In the present study, the level of the p-LIMK1 expression was also downregulated during *Trio* knockdown in human cervical cancer cells.

Invasion and migration are not the only signs of tumor progression, but are also the major reasons behind failures in clinical treatment and patient deaths. Our study revealed the importance of *Trio* in the migration and invasion of cervical cancer. Since all active forms of proteins were reduced in *Trio*-deficient cells, the aberrant cell migration and invasion upon *Trio* knockdown may be results of lower RhoA activity, and impaired migration and invasion possibly arose from the

drop in Rock and p-LIMK1 activity. The *Trio*/RhoA/ROCK pathway regulates the progression of cervical cancer metastasis, and blocking the *Trio* signaling pathway could be a feasible treatment strategy in inhibiting tumor invasion and migration. In conclusion, the present study provided evidence that *Trio* expression was increased in cervical cancer tissue samples, and was closely correlated with lymph node metastasis. Our findings indicate that *Trio* is a promising diagnostic marker for the identification of cervical cancer individuals who are at high risk of lymph node metastasis.

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