Shikonin inhibits the proliferation of cervical cancer cells via FAK/AKT/GSK3β signalling

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Abstract. Cervical cancer is one of the most lethal malignancies of the female reproductive system. Shikonin, a naphthoquinone pigment extracted from the traditional medicinal herb, *Lithospermum erythrorhizon*, has been demonstrated to exert significant inhibitory effects on a variety of tumours *in vitro* and *in vivo*. In the present study, the effects of shikonin on cervical cancer and the underlying mechanisms were investigated. The effects of shikonin on the viability on HeLa and SiHa cervical cancer cells was examined using cell counting kit (CCK-8) and colony formation assays. Immunofluorescence assay was performed to detect the levels of the proliferation-related protein, Ki67. Western blot analysis was utilized to measure the phosphorylated and total expression levels of proteins, including focal adhesion kinase (FAK), AKT, and glycogen synthase kinase 3β (GSK3β). Cell migration was determined by using wound healing assay. Metastasis-associated 1 (MTA1), TGFβ1 and VEGF mRNA expression levels were determined using reverse transcription-quantitative PCR. It was demonstrated that, shikonin inhibited cervical cancer cell proliferation and migration. The data of the present study revealed that shikonin inhibited the proliferation of HeLa and SiHa cells in a concentration- and time-dependent manner. Mechanistically, shikonin blocked the proliferation of cervical cancer cells by downregulating the phosphorylation of FAK, AKT and GSK3β induced by EGF. In addition, shikonin significantly suppressed cell migration and reduced the expression of migration-related proteins, including MTA1, TGFβ1 and VEGF. On the whole, the present study demonstrates that shikonin may exert an inhibitory effect on the cervical cancer cell proliferation and migration through the FAK/AKT/GSK3β signaling pathway. These findings suggest that shikonin may function as a potential therapeutic drug for the treatment of cervical cancer.

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Introduction

Cervical cancer is one of the most common types of cancer among female patients worldwide, with ~530,000 new cases and >274,000 deaths each year (1,2). Although the morbidity and mortality rates of patients with cervical cancer have decreased in most parts of the world over the past few decades, due to the improvement of the average socio-economic levels and the reduced risk of persistent high-risk human papillomavirus infection, cervical cancer remains the most commonly diagnosed type of cancer in low-and middle-income countries, mainly in sub-Saharan Africa (2,3). Particularly in low-income countries, the incidence and mortality rates of cervical cancer are markedly higher, and it is a major cause of cancer-related mortality among female cancer patients (1,4).

Cervical cancer has not been reported to be sensitive to chemotherapeutic drugs in the conventional view. Therefore, surgery and radiotherapy are usually selected for treatment. However, with recent advancements being made in research, numerous experimental results and clinical practices have confirmed that surgery and radiotherapy cannot completely control or eliminate the occurrence and metastasis of cervical

cancer (5). In terms of chemotherapy, cisplatin-based chemotherapy is the most commonly applied regimen; however, cisplatin-based chemotherapy as the main treatment of metastatic cervical cancer does not significantly improve survival (6,7). Therefore, there is an urgent need for improved treatments and more effective therapeutic targets for the treatment of cervical cancer.

Traditional Chinese herbal medicines and their active ingredients have been widely used in clinical practice, due to their reduced toxicity and high efficacy in tumour therapy (8,9). Therefore, the anticancer efficacy of traditional Chinese medicine and its extracts has become a hot topic of research. Among these medicines, shikonin is a naphthoquinone pigment, extracted from the traditional medicinal herb, *Lithospermum erythrorhizon*, which has been reported to exhibit extensive biological activities, particularly anticancer activity (10). It has been demonstrated in a previously published study that the cancer inhibitory effects of shikonin may occur through various mechanisms, including inhibition of cell proliferation and migration, induction of apoptosis and autophagy, and inhibition of glycolysis and metabolism (11). Additionally, it has been revealed that shikonin may block PI3K/Akt and ERK-mediated epithelial-mesenchymal transition (EMT) pathways by inhibiting c-Met, thus preventing HCC827 lung cancer cell migration and invasion and inhibiting the proliferation of HCC827 cells by acting on the EMT transition and HGF (12). Shikonin-containing liposomes have also been revealed to inhibit angiogenesis and induce the downregulation of VEGF gene expression in the human umbilical vein endothelial cells (HUVECs), ultimately inhibiting angiogenesis (13). Other studies have revealed that shikonin may inhibit DNA methyltransferase 1 expression, decrease PTEN gene methylation and increase PTEN protein expression, thereby inhibiting the migration of TPC-1 cells from thyroid cancers (14). However, limited reports have been released on the effects of shikonin on cervical cancer. Previous studies have only focused on the inhibitory effects of shikonin on EMT (15) and the activation of caspase-3 (16) in cervical cancer; thus, the complete mechanisms remain to be explored in depth.

Epidermal growth factor receptor (EGFR) is one of the most frequently overexpressed, amplified and mutated genes in human cancers (17). EGFR regulates tumour proliferation, invasion, apoptosis and angiogenesis through multiple signalling pathways, including PI3K, RAS/RAR/MEK1/ERK1/2 and Janus kinase (JAK)/STAT, in cervical and other cancers (18-20). The focal adhesion kinase (FAK)-mediated signalling pathway, which is dependent on receptor tyrosine kinase (PTK) activity, plays a pivotal role in regulating the occurrence and development of tumours (21). FAK may be phosphorylated after binding to some signalling and cytoskeleton molecules to transmit signals from the extracellular matrix or deliver signals from soluble bioactive factors (22). As it has been reviewed Zhou *et al* (23) FAK may play an indispensable role in tumourigenesis, by consistently promoting proliferation and survival signals.

Of note, it has been revealed that salinomycin, an antitumour drug, may increase cell stiffness and F-actin formation in hepatocellular carcinoma stem cells via the FAK-ERK1/2 pathway, in order to attenuate hepatocellular carcinoma stem cell motility (24). FAK has also been demonstrated to induce inflammatory factor expression, which may suppress antitumour immunity in the microenvironment and ultimately lead to tumour immune escape (25). Those previously published findings may suggest that EGFR and FAK could be potential targets for cancer therapy.

In the present study, it was revealed that shikonin inhibits cervical cancer cell proliferation and migration, probably by inhibiting the EGF-mediated phosphorylation signalling pathway of FAK/AKT/glycogen synthase kinase 3β (GSK3β).

Materials and methods

Reagents. Shikonin was acquired from MedChemExpress (cat. no. HY-N0822, batch 39014, purity >99.80%). PF-562271 (FAK selective inhibitor) (cat. no. HY-10459) was purchased from MedChemExpress. EGF (cat. no. 236-EG-01 M) was purchased from Merck KGaA. HeLa and SiHa cells were pre-treated with PF-562271 (10 μM) for 60 min followed by co-treatment with EGF (10 ng/ml) for 60 min. Reagents used for cell culture, including DMEM and foetal bovine serum (FBS), as well as penicillin and streptomycin, were purchased from Gibco; Thermo Fisher Scientific, Inc. Finally, 2X M5 HiPer SYBR Premix EsTaq (with Tli RNaseH) (cat. no. MF787-01) was purchased from Beijing Jumei Biotechnology Co., Ltd.

Cells and cell culture. HeLa and SiHa human cervical cancer cells (iCell Bioscience Inc.) were cultured in Dulbecco's modified Eagle's medium (DMEM) containing 4.5 g/l glucose supplemented with 10% foetal bovine serum (Gibco; Thermo Fisher Scientific, Inc.). Cells were maintained at 37˚C in an incubator with 5% CO_2. When the degree of cell confluence reached 85-90%, the cells were sub-cultured. All cell lines were found to be free of mycoplasma (data not shown).

Measurement of cell viability. Cell viability assays were performed, using the Cell Counting Kit-8 (CCK-8; Dojindo Laboratories, Inc.) as previously described (26). Briefly, cells were seeded in culture medium in 96-well plates ($2.0x10^4$ cells/ml; 100 μl). The wells without cells served as the blank control. Following attachment, the cells were serum-starved overnight and incubated at 37˚C with various concentrations of shikonin for 24, 48 and 72 h. The concentration range (1-4 μM) was selected according to previous studies, which have revealed that shikonin can effectively inhibit cell proliferation at this range in oesophageal cancer (27,28). Subsequently, 10 μl CCK-8 solution were added to each well. The optical density (OD) at 450 nm was assayed by spectrophotometer (type 1530; Thermo Fisher Scientific, Inc.) after cell incubation at 37˚C for 2 h. The IC50 values of shikonin on cell viability were calculated by non-linear regression, using GraphPad Software Prism 7.0 (GraphPad Software, Inc.) (29).

Western blot analysis. Cells were cultured in serum-free DMEM medium overnight for shikonin treatment. The concentration of shikonin used was 2.5 μM, according to the concentration-related experiments and previously published studies (27,30). Following treatment, all cells were incubated with cell lysis buffer (cat. no. R0010; Beijing Solarbio Science & Technology Co., Ltd.) containing protease inhibitors (cat. no. 04 693 132 001; Roche Diagnostics GmbH) and phosphatase inhibitors (cat. no. 04 906 837 001; Roche Diagnostics GmbH) at 4˚C for 10 min. The

Table I. Primer pair sequences.

Gene name	Primer sequences (5'-3')
MTA1	Forward: 5'-CATCAGAGGCCAACCTTTTCG-3'
	Reverse: 5'-GCACGTATCTGTCGGTGGTC-3'
TGFβ1	Forward: 5'-ACTCTCTGACTTCCGCGTTC-3'
	Reverse: 5'-CACTTGCCCAGCAATAGGTTTAT-3'
VEGF	Forward: 5'-CTGGGCTGTTCTCGCTTCG-3'
	Reverse: 5'-CTCTCCTCTTCCTTCTCTTCTTCC-3'
GAPDH	Forward: 5'-ACAACTTTGGTATCGTGGAAGG-3'
	Reverse: 5'-GCCATCACGCCACAGTTTC-3'

MTA1, metastasis-associated 1; Fw, forward; Rv, reverse.

protein concentration was measured using the BCA protein assay kit (cat. no. P0010; Beyotime Institute of Biotechnology). Equal amounts of protein (10 μg/lane) were loaded and separated by 10% SDS-PAGE. Proteins were transferred to PVDF membranes (cat. no. IPVH00010; EMD Millipore) and blocked in 5% non-fat dry milk for 2 h, at room temperature. Subsequently, the membranes were incubated overnight at 4˚C with the following primary antibodies: Anti-phospho-FAK (Tyr397) (cat. no. 8556; 1:2,000), anti-FAK (cat. no. 71433; 1:2,000), anti-phospho-AKT (Ser473) (cat. no. 4060; 1:2,000), anti-AKT (cat. no. 4685; 1:2,000), β-actin (cat. no. 4970; rabbit anti-human; 1:5,000), anti-phospho-GSK3β (Ser9) (cat. no. 9323; 1:2,000) and anti-GSK3β (cat. no. 9315; 1:2,000). The membranes were then incubated with HRP-conjugated anti-rabbit IgG secondary antibody (cat. no. 7074; goat anti-rabbit; 1:4,000) for 2 h at room temperature. All primary and secondary antibodies were bought from Cell Signaling Technology, Inc. After washing the membrane with TBST (0.1% Tween-20), enhanced chemiluminescence reagent (Pierce™ ECL Western Blotting Substrate; cat. no. 32106; Thermo Fisher Scientific, Inc.) was added. ImageJ software (version 1.47t; National Institutes of Health) was used to analyse the grey value of each target band, which indicated the expression level of the target protein as compared with that of β-actin.

Wound healing assay. The wound healing assay was performed to measure the effects of shikonin on cell migration according to previous studies (31,32). Briefly, the cells were seeded in a 6-well plate at a concentration of $4.5x10^5$ cells/well and were grown until reaching 90% confluency. The bottoms of the 6-well plates were marked, and the wounds were scratched vertically with a 200 μl pipette tip. After washing three times with phosphate-buffered saline (PBS; cat. no. KGB5001; KeyGEN BioTECH, Inc.), the cells were treated with shikonin and incubated at 37˚C in DMEM (Gibco; Thermo Fisher Scientific, Inc.) with 2% FBS (Gibco; Thermo Fisher Scientific, Inc.) for 24 and 48 h. The representative scrape lines for each set were photographed using a phase-contrast microscope (DMi1; Leica Microsystems) at 0, 24 and 48 h.

Immunofluorescence confocal microscopy. The immunofluorescence assay was performed as previously described (26). Briefly, $6x10^4$ cells per well were seeded on coverslips in 12-well plates for 24 h. Following drug treatment, the cells were fixed with 4% paraformaldehyde for 20 min at room temperature, permeabilized with 0.3% Triton X-100 (cat. no. P0096; Beyotime Institute of Biotechnology) in PBS for 15 min, and blocked in PBST (0.1% Tween-20) containing 5% bovine serum albumin. Thereafter, the cells were incubated with anti-human rabbit Ki67 antibody (cat. no. 27309-1-AP; 1:200, ProteinTech, Group, Inc.) overnight at 4˚C. The cells were then washed three times with ice-cold PBS, followed by incubation with the anti-rabbit secondary antibody [Goat Anti-Rabbit IgG H&L (Alexa Fluor® 594); cat. no. ab150080; 1:300; Abcam] for 60 min at room temperature. DAPI (cat. no. AR1177; Wuhan Boster Biological Technology, Ltd.) was used for nuclear staining at room temperature for 10 min. Finally, images were captured with a fluorescence microscope (IX83; Olympus Corporation) and quantified with Image-Pro Plus 6.0 software (Media Cybernetics, Inc.).

Colony formation assay. In total, 200 HeLa and SiHa cells per well seeded into 6-well plates were cultured for 5-7 days until small colonies could be clearly observed. Cells were treated with various concentrations (0, 2.5 and 3.5 μM) of shikonin in DMEM (Gibco; Thermo Fisher Scientific, Inc.) for 8-12 days. Subsequently, cells were fixed with 4% formaldehyde (Sigma-Aldrich; Merck KGaA) for 15 min. The colonies were stained with 0.2% crystal violet solution (Sigma-Aldrich; Merck KGaA) in 10% ethanol for 10 min. Excess stain was removed by washing repeatedly with PBS. All procedures were conducted at room temperature. Image-Pro Plus 6.0 software (Media Cybernetics, Inc.) was used for quantification.

Reverse transcription-quantitative PCR (RT-qPCR). The RT-qPCR assay was performed as previously described (33,34). Briefly, total cellular RNA was extracted using TRIzol® reagent (cat. no. 15596018; Invitrogen; Thermo Fisher Scientific, Inc.). According to the manufacturer's instructions, total RNA was reverse transcribed into cDNA using the Thermo Scientific™ RevertAid™ First Strand cDNA Synthesis kit (cat. no. K1621; Thermo Fisher Scientific, Inc.) in a thermal cycler (Mastercycler® Nexus; cat. no. 6331000076; Eppendorf) at the following temperatures: 40˚C for 60 min and 70˚C for 5 min. The PCR primer sequences are presented in Table I. qPCR was performed

using 2X M5 HiPer SYBR Premix EsTaq (Beijing Jumei Biotechnology Co., Ltd.; cat. no. MF787-01), the 20-μl PCR system contained 2 μl cDNA, 10 μl 2X M5 HiPer SYBR Premix EsTaq (with Tli RNaseH), 0.4 μl ROX Reference Dye II, 0.4 μl 10 μM Primer 1, 0.4 μl 10 μM Primer 2 and 6.8 μl ddH_2O. The qPCR amplification was performed using the ViiA7 system (Applied Biosystems; Thermo Fisher Scientific, Inc.) under the following thermocycler conditions: 95˚C for 30 sec, 95˚C for 3 sec and 60˚C for 30 sec for 40 cycles. The relative gene expression levels were calculated using the $2^{-\Delta\Delta Cq}$ method and normalized to GAPDH (35). At least three independent experiments were conducted, and samples were evaluated in triplicate in each experiment.

Statistical analysis. Where indicated, data are represented as the mean ± SEM and were analysed by using GraphPad Software Prism 7.0 (GraphPad Software, Inc.). Comparisons were made using one/two-way ANOVA with Bonferroni's multiple comparison tests (when more than two groups were compared). $P<0.05$ was considered to indicate a statistically significant difference.

Results

Shikonin inhibits the growth of cervical cancer cells. To detect the effect of shikonin on the cell growth of cervical cancer cells, various concentrations of shikonin were added to the HeLa and SiHa cervical cancer cell lines. Following incubation for 48 h, cell viability was assessed using a CCK-8 assay. The results revealed that shikonin inhibited HeLa and SiHa cell proliferation in a concentration-dependent manner, with IC50 values of ~2.9 μM in HeLa cells and 2.2 μM in SiHa cells (Fig. 1A and G). To further examine the effects of different treatment times on the cell proliferation, HeLa and SiHa cells were treated with Shikonin for 24, 48 and 72 h at concentrations of 0, 1.5, 2.5 and 3.5 μM. The results demonstrated that the inhibitory effect of shikonin on cell proliferation became increasingly obvious with prolonged administration time (Fig. 1B and H). Taken together, these results suggest that shikonin markedly inhibits the proliferation of cervical cancer cells in a concentration- and time-dependent manner.

Ki67 is a nuclear antigen related to cell proliferation and it is a critical biomarker for tumour cell proliferation (36). In the present study, HeLa and SiHa cells were then treated with shikonin for 48 h and the expression of Ki67 was detected using immunofluorescence assay. As depicted in Fig. 1C and I, shikonin treatment markedly decreased the number and fluorescence intensity of Ki67-positive cells. The results indicated that shikonin exerted a significant inhibitory effect on the proliferation of HeLa and SiHa cervical cancer cells (Fig. 1C, D, I and J).

To further confirm the aforementioned findings, a colony formation assay was then performed, in order to evaluate cell proliferation. The results revealed that shikonin significantly inhibited the size of HeLa and SiHa cervical cancer cell colonies. By increasing the drug concentration, the spaces between cells became larger, and the shape of the clonogenic bodies became increasingly smaller (Fig. 1E, F, K and L).

Shikonin reduces the phosphorylation levels of FAK, AKT and GSK3β in cervical cancer cells. FAK is a key regulator of growth factor receptor and integrin-mediated signalling, regulating the basic processes of cancer cell proliferation, migration, invasion and apoptosis through its kinase activity and scaffolding function (37). Similarly, AKT and GSK3β also play a vital role in the growth of tumour cells (38,39). To evaluate whether FAK, AKT and GSK3β are involved in the effects of shikonin, the FAK, AKT and GSK3β phosphorylation levels in cervical cancer cells were measured, following shikonin treatment. It was observed that shikonin significantly inhibited FAK, AKT and GSK3β phosphorylation without any changes in FAK, AKT and GSK3β total protein expression (Fig. 2), suggesting that shikonin inhibits the proliferation of cervical cancer cells through FAK/AKT/GSK3β signalling.

Shikonin blocks the EGF-induced FAK/AKT signalling pathway in cervical cancer cells. Since EGF/EGFR signalling is overactivated in multiple human types of cancer (18), it contributes to tumour proliferation, invasion, apoptosis and angiogenesis. Subsequently, in the present study, the effects of shikonin on EGF-induced FAK, Akt and GSK3β phosphorylation were explored. The cells were pre-treated with shikonin and then stimulated with EGF. As demonstrated in Fig. 3A, EGF upregulated the phosphorylation of FAK, AKT and GSK3β, while shikonin pre-treatment significantly blocked the EGF-induced FAK, AKT and GSK3β phosphorylation (Fig. 3A, B, E and F). Subsequently, to determine whether AKT serves as downstream signalling target of FAK, the effect of PF-562271, a selective inhibitor of FAK, on EGF-induced AKT phosphorylation in HeLa and SiHa cells was detected. All cells were pre-treated with PF-562271 (10 μM) for 60 min and then the cells were treated with EGF (10 ng/ml) for 60 min. It was revealed that PF-562271 markedly inhibited the basal and EGF-induced AKT phosphorylation levels (Fig. 3C, D, G and H), indicating that AKT is a downstream factor of FAK. Therefore, it was hypothesized that the inhibitory effect of shikonin may be also mediated through the FAK/AKT/GSK3β pathway.

Shikonin inhibits the EGFR-induced proliferation of cervical cancer cells through the FAK/AKT/GSK3β pathway. To further validate the results, the present study then whether shikonin inhibits the proliferation of cervical cancer cells induced by EGF. The cells were pre-treated with shikonin and then stimulated with EGF. The results revealed that EGF significantly promoted the proliferation of cervical cancer cells, while shikonin significantly inhibited this effect (Fig. 4A and B). Consistent with the results of western blot analysis, the blocking of FAK using PF-562271 also markedly inhibited the EGF-induced proliferation of cervical cancer cells (Fig. 4C and D). Taken together, these results indicated that shikonin inhibited the proliferation of cervical cancer cells via the FAK/AKT/GSK3β signalling pathway.

Shikonin inhibits cell migration and decreases the expression level of related proteins. Cell migration plays an essential role in tumour progression. Thus, to determine whether shikonin has any effect on the migration of cervical

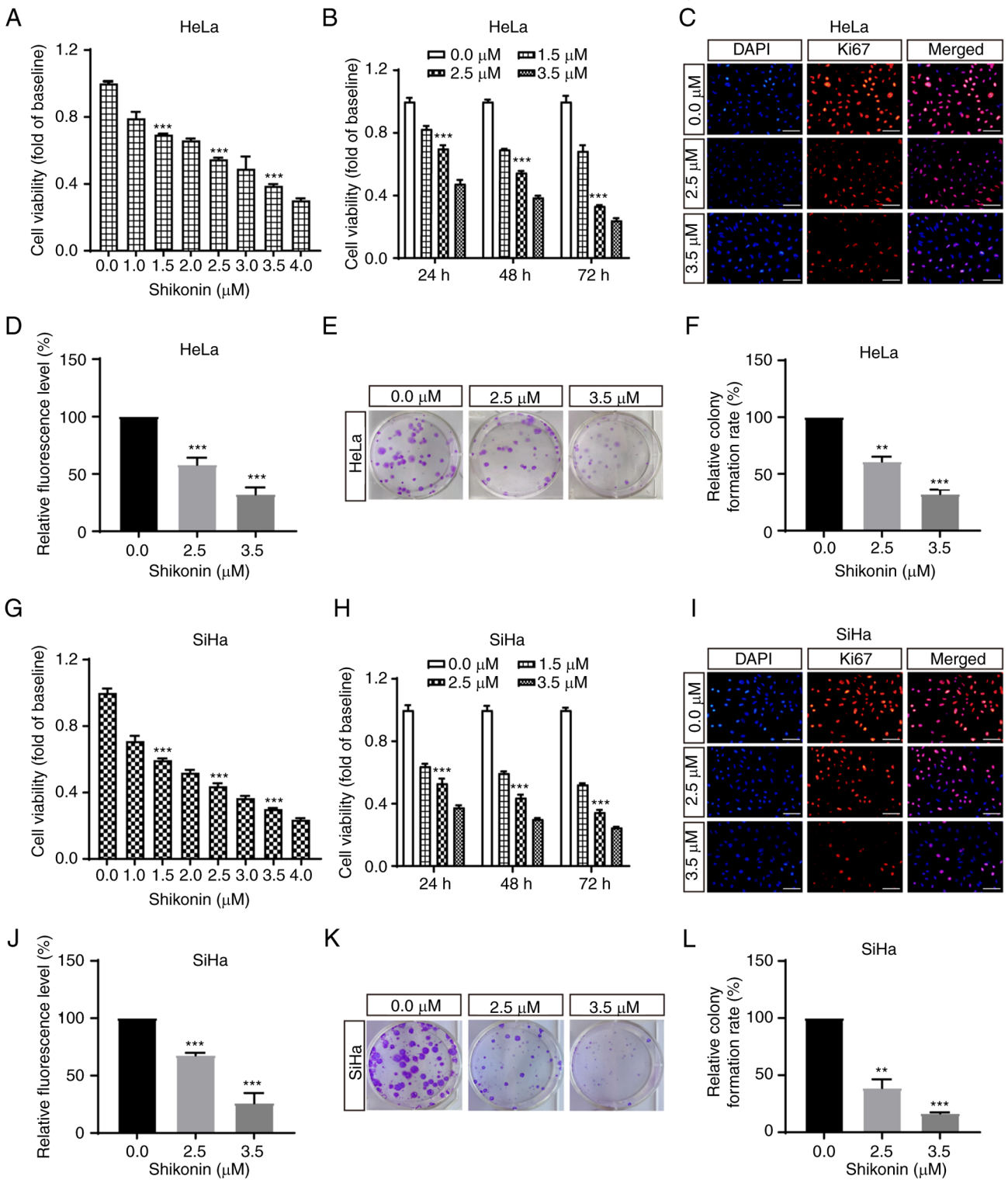

Figure 1. Shikonin inhibits the proliferation and clonogenicity of cervical cancer cells. (A and G) Concentration-dependent effects of shikonin on the proliferation of cervical cancer cells. (A) HeLa and (G) SiHa cells were treated with increasing concentrations of shikonin as indicated, for 48 h. (B and H) Time-dependent effects of shikonin on the proliferation of cervical cancer cells. (B) HeLa and (H) SiHa cells were treated with shikonin for 24, 48 and 72 h. (C and I) Immunofluorescence confocal microscopy was used to assess the expression of Ki67 in cervical cancer cells after shikonin treatment. Scale bar, 50 μm, 20X objective. (D and J) Quantification of the fluorescence level as depicted in C&I. (E and K) Colony formation experiment of SiHa and HeLa cells, following incubation with shikonin at various concentrations. (F and L) Quantification of the colony formation results revealed in (E and K). Values represent the mean ± SEM. **P<0.01 and ***P<0.001 vs. the control group. SEM, standard error of the mean

cancer cells, a wound healing assay was then performed to evaluate the effects of shikonin on HeLa and SiHa cell metastatic capability. Notably, shikonin significantly inhibited the migration of cervical cancer cells (Fig. 5A-D). Numerous studies have demonstrated that metastasis-associated 1 (MTA1) (40,41), TGFβ1 (42) and VEGF (43) play crucial

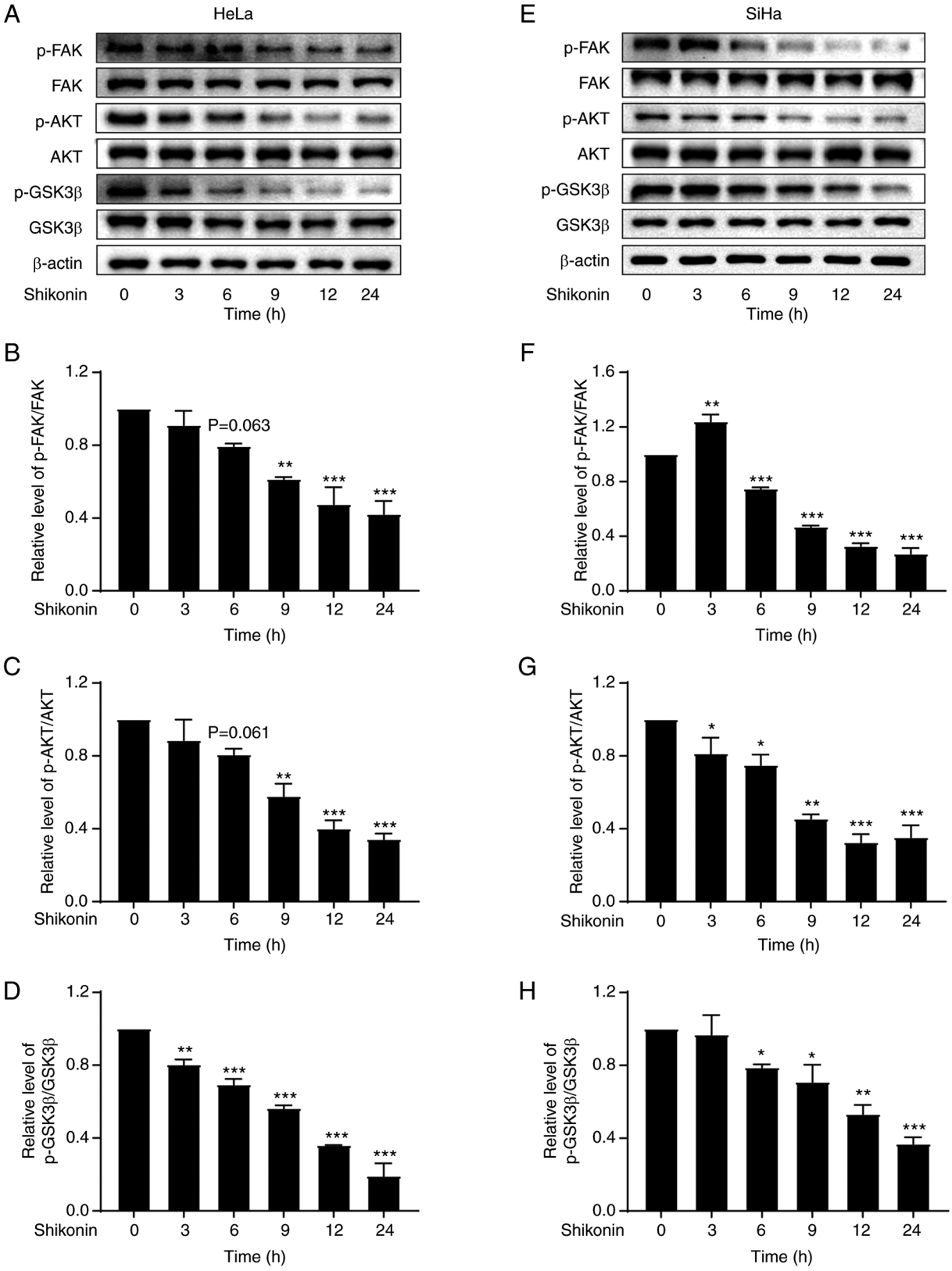

Figure 2. Shikonin reduces the phosphorylation levels of FAK, AKT and GSK3β in cervical cancer cells. (A and E) Phosphorylation levels of FAK, AKT, and GSK3β following treatment with 2.5 μM shikonin for different periods of time in (A) HeLa and (E) SiHa cells, as measured using western blot analysis. (B-D and F-H) Quantification of the western blot analysis results. Values represent the mean ± SEM. *P<0.05, **P<0.01, ***P<0.001 vs. the control group. FAK, focal adhesion kinase; SEM, standard error of the mean.

roles in the migration of cervical cancer cells. Thus, the present study then detected the transcription levels of these genes. As shown in Fig. 5E, shikonin markedly inhibited the expression of MTA1, TGFβ1 and VEGF in HeLa cells (Fig. 5E), further confirming the inhibitory effects of shikonin on cervical cancer cells.

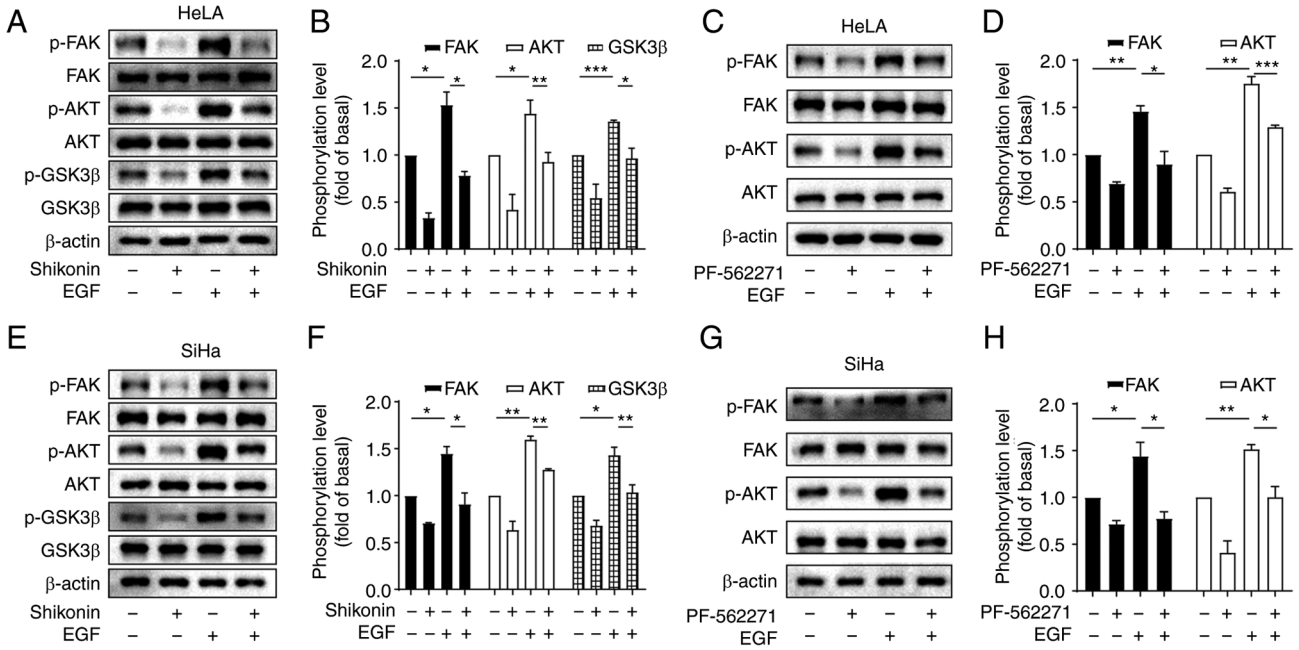

Figure 3. Shikonin blocks the EGF-induced FAK/AKT signalling pathway in cervical cancer cells. (A and E) Effects of shikonin on EGF-induced FAK and AKT phosphorylation in HeLa and SiHa cells. (B and F) Quantification of western blot analysis results. (C and G) Effects of PF-562271 on EGF-induced FAK and AKT phosphorylation in HeLa and SiHa cells. (D and H) Quantification of western blot analysis results. Values represent the mean ± SEM. *P<0.05, **P<0.01, ***P<0.001 vs. the control group. FAK, focal adhesion kinase; SEM, standard error of the mean.

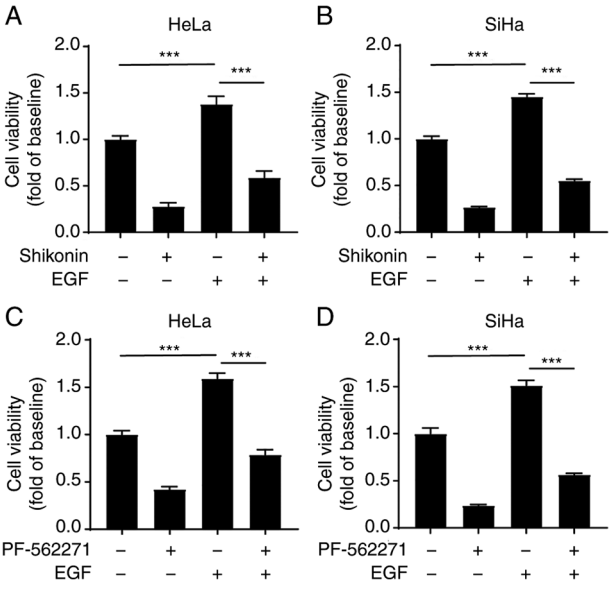

Figure 4. Shikonin inhibits the EGFR-induced proliferation of cervical cancer cells through the FAK/AKT/GSK3β pathway. (A and B) HeLa and SiHa cells were treated with shikonin (2.5 μM) in the absence or presence of EGF (10 ng/ml) for 72 h, and then cell viability was evaluated by using CCK-8 assay. (C and D) HeLa and SiHa cells were treated with PF-562271 (10 μM) in the absence or presence of EGF (10 ng/ml) for 72 h, and then cell viability was measured by CCK-8 assay. Values represent the mean ± SEM. ***P<0.001 vs. the control group. FAK, focal adhesion kinase; SEM, standard error of the mean.

Discussion

A recent review article revealed that shikonin exerts a significant inhibitory effect on the occurrence of numerous tumours (44); however, its effects and mechanisms of action in cervical cancer remain largely unknown. In the present study, it was revealed that shikonin inhibited the proliferation of HeLa and SiHa cervical cancer cells, and this effect may be mediated through the downregulation of FAK/AKT/GSK3β phosphorylation. In addition, shikonin significantly inhibited the migration of cervical cancer cells and reduced the transcriptional levels of the MTA1, TGFβ1 and VEGF genes.

Shikonin has been reported to exert antitumour effects on a variety of tumours, such as lung, breast, gastrointestinal and pancreatic cancer (44). In the present study, it was demonstrated that shikonin inhibited the viability of HeLa and SiHa cervical cancer cells in a concentration- and time-dependent manner. This was further supported by evidence that shikonin inhibited the expression of Ki67, a widely used biomarker for cell proliferation of tumour cells. In addition, the colony formation assay also revealed that the number of colonies was significantly reduced following shikonin treatment. All these findings by different assays consistently underline that shikonin may exert a significant inhibitory effect on the proliferation of HeLa and SiHa cervical cancer cells. One of the limitations of the present study was that the effect of shikonin on a non-cancerous cervical cell line was not examined. Notably, a recent study indicated that shikonin may exert a general anticancer effect and a relatively less prominent inhibitory effect on normal cells (29). Furthermore, a clinical trial study reported that the shikonin mixture was safe and effective in the treatment of patients with late-stage lung cancer who were unsuitable for radiotherapy, chemotherapy and surgery; more importantly, shikonin treatment had no harmful effects on peripheral system, heart, kidney and liver and even increased the body weight and appetite of the patients (45). Another limitation of the present study is that although this study was

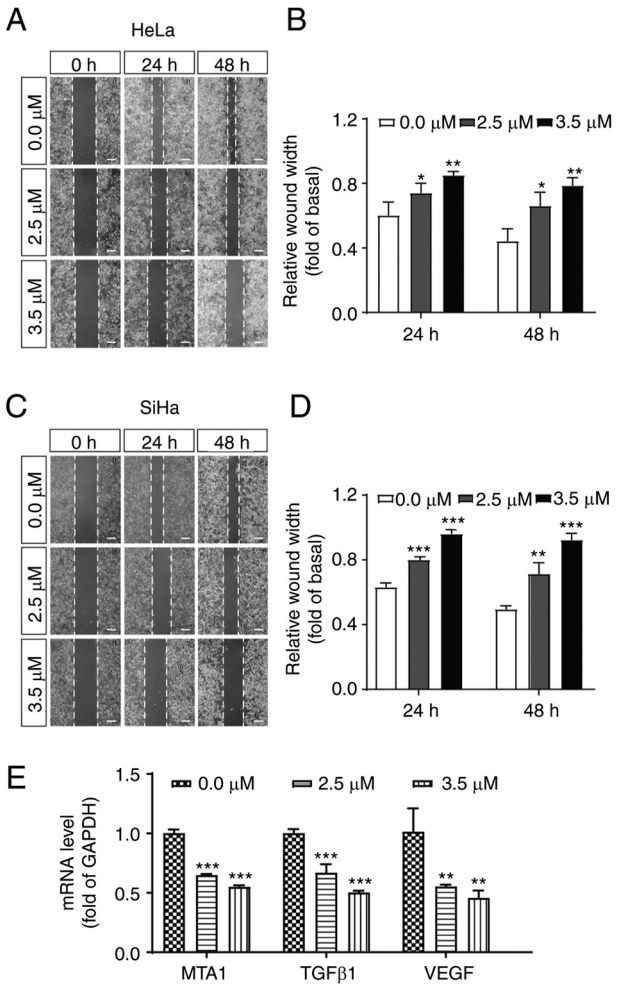

Figure 5. Shikonin suppresses cell migration and decreases the expression level of related proteins. (A and C) Cells were treated with shikonin (0 μM, 2.5 μM and 3.5 μM), and the effects of shikonin on migration were measured by using wound healing assay. Scale bar, 250 μm. (B and D) The relative wound width was measured in at least three wounds and normalized to the control. (E) Effects of shikonin on MTA1, TGFβ1 and VEGF in HeLa cells. Values represent the mean ± SEM. *P<0.05, **P<0.01 and ***P<0.001 vs. the control group. MTA1, metastasis-associated 1; SEM, standard error of the mean.

mainly focused on the effects of shikonin on cell proliferation, whether shikonin exerts its anti-proliferative effect on cervical cancer due to cell cycle arrest, death and/or apoptosis remains unknown. Of note, it has previously been reported that shikonin inhibits the proliferation of AGS human stomach carcinoma cells by inducing apoptotic cell death (46). Shikonin has been revealed to arrest the cell cycle in the G2/M phase, inhibit cell growth and induce cell death, which collectively contributes to the growth inhibitory effects of shikonin in various cancer cell lines originating from lung, breast, pancreas, osteosarcoma, while its inhibitory effects on normal cells are more limited in comparison (29). Thus, the effects of shikonin on cell cycle progression and the apoptosis of cervical cancer require further investigation.

FAK is an important regulatory molecule in growth factor receptors and integral protein-mediated signalling pathways (47). In multiple types of tumours, the expression of activated FAK has been reported to be significantly increased (48). The overexpression and hyperphosphorylation of FAK have been revealed to promote cancer cell proliferation, motility and survival (49). Of note, in the present study, it was observed that shikonin inhibited FAK phosphorylation levels in cervical cancer cells. AKT is a serine/threonine protein kinase that plays a crucial role in regulating cell survival and apoptosis, and studies have revealed that AKT activation can affect cell growth, proliferation, migration, apoptosis and angiogenesis of tumour cells by regulating many downstream proteins (50). In addition, several studies have demonstrated that the PI3K/AKT/GSK3β signalling pathway may regulate epithelial-mesenchymal transition in numerous tumours, thus participating in tumour invasion and metastasis (51-53). However, the FAK/AKT/GSK3β signalling pathway has not, to the best of our knowledge, been previously studied in cervical carcinogenesis. In the present study, it was demonstrated that shikonin significantly inhibited the phosphorylation levels of FAK, AKT and GSK3β in cervical cancer cells, which is a different mechanism in comparison to the signalling pathways reported concerning other tumour types. For example, Tang *et al* (27) demonstrated that shikonin inhibited the proliferation of oesophageal cancer cells, which is associated with the HIF1α/PKM signalling pathway. Shan *et al* (54) revealed that shikonin suppress human leukemia NB4 cell proliferation and induce apoptosis by regulating MAPKs and c-Myc. Pan *et al* (55) reported that shikonin blocked human lung adenocarcinoma development through the IL-6/STAT3 signalling pathway. The mechanisms by which shikonin functions to block the phosphorylation of FAK/AKT remain largely unknown. Previous studies have revealed that the FAK/AKT axis is activated by receptor tyrosine kinases, including EGFR and IGF-1R. However, there are few reports on the exact molecular mechanisms through which these receptors regulate FAK phosphorylation in tumours, and previous studies have reported that these receptors may regulate the FAK/AKT pathway through Src and NF-KB phosphorylation (56,57).

EGFR is an important tyrosine kinase receptor in tumourigenesis and has been reported to play a crucial role in tumour cell proliferation, migration and apoptosis (58). In the present study, to further investigate the underlying mechanisms through which shikonin inhibits the proliferation of cervical cancer cells, the effects of shikonin on the EGFR-induced signalling pathway were first detected. EGF significantly increased the phosphorylation levels of FAK and AKT, which were markedly decreased by shikonin treatment. However, compared to shikonin alone, EGF still increased the phosphorylation level of FAK and AKT in the presence of shikonin, indicating shikonin may not completely block EGF-induced activation of downstream signalling cascades. There are two possible explanations for these findings. On the one hand, the concentration of shikonin may markedly low; thus, it can only partially block the EGF-induced effect. Firstly, EGF may also induce FAK and AKT phosphorylation through other signalling cascades that are insensitive to shikonin. It is worth noting that since the mechanisms through which shikonin blocks FAK/AKT phosphorylation and affect the phosphorylation and total expression levels of EGFR remain unknown, the exact mechanisms involved need to be further investigated.

Notably, the EGF-induced increase in AKT phosphorylation could be significantly inhibited by the FAK-specific blocker, PF-562271. Therefore, it can be inferred that AKT

may be the downstream signalling molecule of FAK. In addition, cervical cancer cells were treated with EGF in the absence or presence of PF-562271 and it was observed that PF-562271 inhibited the EGF-induced increase in FAK and AKT phosphorylation levels. Taken together with the CCK-8 assay findings, demonstrating that PF-562271 significantly inhibited EGF-induced cell proliferation, it was speculated that the inhibition of FAK phosphorylation may cause a decrease in downstream AKT phosphorylation, in turn weakening the proliferation of cervical cancer cells. Thus, these findings indicated that shikonin-induced inhibition of cervical carcinogenesis may be mediated through the FAK/AKT/GSK3β phosphorylation signalling pathway.

Another important finding of the present study is that shikonin also suppressed the cell migration of cervical cancer cells, as evaluated using wound healing assay. Moreover, shikonin inhibited the expression of migration-related genes, including MTA1, TGFβ1 and VEGF, suggesting that shikonin may inhibit the proliferation of tumour cells by affecting downstream related genes after inhibiting the phosphorylation of FAK and AKT. In addition, in the future, the authors aim to further validate the tumour suppressive effects in mouse models with subcutaneous tumours or *in situ* cervical cancer *in vivo*. The authors also aim to use mouse tumour tissues for protein, RNA and immunohistochemical staining evaluation, in order to detect the expression of p-FAK, p-AKT and Ki67 proteins to further elucidate the molecular mechanisms involved.

In conclusion, it was observed that shikonin significantly inhibited the proliferation and migration of cervical cancer cells. It was also clarified that shikonin inhibited the proliferation of cervical cancer cells, possibly by regulating the EFG-mediated phosphorylation of the FAK/AKT/GSK3βsignalling pathway. The findings of the present study suggest that shikonin may be a potential drug for use in the clinical treatment of cervical cancer, and provide a theoretical basis for the development of novel therapeutic drugs in the clinical setting.

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Availability of data and materials

The datasets used and/or analysed during the current study are available from the corresponding author on reasonable request.

Authors' contributions

NL, PH and ZX designed the study. ZX and LH conducted the experiments. TZ, YL, FF and XW analysed most of the experimental data. WC, LL and YZ analysed the western blot analysis data. ZX and PH wrote the manuscript. All authors have read and approved the final manuscript. NL and PH confirm the authenticity of all the raw data.

Ethics approval and consent to participate

Not applicable.

Patient consent for publication

Not applicable.

Competing interests

The authors declare that they have no competing interests.

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