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 common female cancers worldwide, and it is one of the most lethal malignancies of the female reproductive system. Shikonin, a natural pigment of theophyllin, has a variety of biological activities and has shown significant inhibitory effects on a variety of tumours in vitro and in vivo. However, there are few studies on Shikonin in cervical cancer. In the present study, we found that Shikonin inhibited not only the proliferation but also the migration of cervical cancer cells. Our data showed that Shikonin inhibited the proliferation of HeLa and SiHa cells in a concentration- and time-dependent manner. In cervical cancer cells, Shikonin not only inhibited the phosphorylation of FAK, AKT and GSK3β but also inhibited the phosphorylation of FAK, AKT and GSK3β induced by EGF. Further exploring the mechanism, we found that Shikonin could inhibit the proliferation of cervical cancer cells by regulating the phosphorylation of the FAK/AKT/GSK3β pathway. In addition, Shikonin significantly inhibited cell migration and reduced the expression of proteins such as MTA1, TGFβ1 and VEGF. In conclusion, our study elucidated that Shikonin has an inhibitory effect on the proliferation and migration of cervical cancer cells, which may be mediated by the FAK/AKT/GSK3β signalling pathway. Our results suggest that Shikonin has the potential to become a clinical treatment for cervical cancer.

Introduction

Cervical cancer is still the most common cancer among women worldwide, with approximately 530,000 new cases and over 274,000 deaths each year[1, 2]. Although the incidence of cervical cancer in the United States has been declining due to vaccination and screening efforts, it is still far from being eradicated in developed countries. Particularly in resource-poor countries, the cervical cancer incidence and mortality rates are higher and it is one of the leading causes of cancer-related deaths in women[2, 3].

Cervical cancer is not sensitive to chemotherapeutic drugs in the conventional view. Therefore, surgery and radiotherapy are usually chosen for treatment. However, with advances in research, many experimental results and clinical practice have confirmed that surgery and radiotherapy cannot completely control or eliminate the occurrence and metastasis of cervical cancer. In terms of chemotherapy, cisplatin-based chemotherapy is the most commonly applied regimen, but cisplatin-based chemotherapy as the main treatment of metastatic cervical cancer does not significantly improve survival[4]. Therefore, better treatments and more effective therapeutic targets is an urgent need in the treatment of cervical cancer today. Traditional Chinese herbal medicines and their active ingredients are widely used in clinical practice because of their low toxicity, strong specificity and high efficacy in the treatment of tumours[5]. Therefore, the anticancer efficacy of traditional Chinese medicine and its extracts has become a hot topic of research.

Shikonin is the main active component of the traditional Chinese medicine Comfrey, which has extensive biological activities, especially anticancer activity[6]. Many studies have shown that the cancer inhibitory effects of Shikonin can occur through a variety of mechanisms, including inhibition of cell proliferation and migration, induction of apoptosis and autophagy, and inhibition of glycolysis and metabolism[7]. It’s

found that in different tumour cell lines, such as lung, breast and pancreas, Shikonin could act as a potent cell cycle inhibitor and block cells in G2/M phase by upregulating P21[8]. Meanwhile, HSIEH et al. found that Shikonin blocked the PI3K/Akt and ERK-mediated epithelial-mesenchymal transition (EMT) pathways by inhibiting c-Met, thus preventing the migration and invasion of HCC827 lung cancer cells and inhibiting the proliferation of HCC827 cells by acting on the EMT transition and HGF[9]. Shikonin and its liposomes were also found to inhibit angiogenesis and to have a downregulatory effect on VEGF gene expression in the human umbilical vein endothelial cell line HUVEC, which inhibited angiogenesis[10]. Other studies have shown that Shikonin can inhibit DNMT1 expression, decrease PTEN gene methylation and increase PTEN protein expression, thereby inhibiting the migration of TPC-1 positive cells from thyroid cancers[11]. However, there are few reports on the effect of Shikonin on cervical cancer. Previous studies have only focused on the inhibitory effects of Shikonin on EMT[12] and the activation of caspase-3[13], with the complete mechanism still to be explored in depth.

Epidermal growth factor receptor (EGFR) is one of the most frequently overexpressed, amplified and mutated genes in human cancer[14]. EGFR regulates tumour proliferation, invasion, apoptosis and angiogenesis through multiple signalling pathways, including PI3K, RAS/RAR/MEK1/ERK1/2 and JAK/STAT[15]. The focal adhesion kinase (FAK)-mediated signalling pathway, which is dependent on protein tyrosine kinase (PTK) activity, is one of the most important signal transduction pathways in the extracellular matrix[16] and is closely related to the occurrence and development of tumours. FAK can be phosphorylated after binding to some signalling and cytoskeleton molecules to transmit signals from the extracellular matrix (ECM) or to deliver signals from soluble bioactive factors[17]. Several studies have shown that FAK plays an indispensable role in tumorigenesis by consistently promoting proliferation and survival signals[18]. Interestingly, it has been shown that salinomycin, an antitumour drug, can increase cell stiffness and F-actin formation in hepatocellular carcinoma stem cells (LCSCs) via the FAK-ERK1/2 pathway to attenuate hepatocellular carcinoma stem cell motility[19]. At the same time, FAK has been shown to induce the expression of inflammatory genes, the products of which can suppress antitumour immunity in the microenvironment and lead to the immune escape of tumours[20]. These results suggest that EGFR and FAK may be potential targets for cancer therapy.

In this study, we found that Shikonin inhibited the proliferation and migration of cervical cancer cells, probably by inhibiting the EGF-mediated phosphorylation signalling pathway of FAK/AKT/GSK3β.

Materials And Methods

Antibodies and reagents. Shikonin was purchased from MedChemExpress (Shanghai, China). Antibodies for western blot analysis, including anti-phospho-FAK (Tyr397) (cat. no. 8556), anti-FAK (cat. no. 71433), anti-phospho-AKT (Ser473) (cat. no. 4060), anti-AKT (cat. no. 4685), anti-β-actin (cat. no. 4970, monoclonal), rabbit anti-human (1:5,000), and anti-rabbit IgG HRP-linked antibody (cat. no. 7074, goat anti-rabbit, 1:5,000) were purchased from Cell Signaling Technology (Beverly, MA, USA). PF-562271 (FAK selective inhibitor) (cat. no. HY-10459) was purchased from MedChemExpress (Shanghai, China). EGF (cat. no. 236-EG-01 M) was purchased from Sigma Aldrich (Shanghai, China). Reagents used for cell

culture, including DMEM and foetal bovine serum (FBS), as well as other reagents, were purchased from Gibco (Thermo Fisher Scientific, Inc.). SYBR® Premix Ex Taq™ II was purchased from Takara Bio, Inc.

Cell culture. HeLa and SiHa human cervical cancer cells (iCell Bioscience Inc,Shanghai, China) were grown in Dulbecco's modified Eagle's medium (DMEM) containing 4.5 g/l glucose supplemented with 10% foetal bovine serum. 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 subcultured.

Measurement of cell viability. Cell viability assays were performed using the Cell Counting Kit-8 (CCK-8; Dojindo Molecular Technologies, Kumamoto, Japan). Cells were seeded in culture medium in 96-well plates (2.0×10^4 cells/mL; 100 μL). The wells without cells served as the blank. After attachment, the cells were serum-starved overnight and incubated with different concentrations of Shikonin. Then, 10μL of CCK-8 solution was added to each well. The optical density (OD) at 450 nm was assayed after cell incubation at 37°C for 2 h.

Western blot analysis. Cells were cultured in serum-free DMEM overnight for drug treatment. After treatment, the cells were incubated with cell lysis buffer at 4°C for 10 min. Equal amounts of protein (10 μg/lane) were loaded and separated by 10% SDS–PAGE. Proteins were transferred to PVDF membranes and blocked in 5% nonfat dry milk for 2 h at room temperature. Subsequently, the membrane was incubated with the indicated antibodies overnight at 4°C and then incubated with HRP-conjugated anti-rabbit IgG secondary antibody (1:4000) for 2 h at room temperature. After washing the membrane, enhanced chemiluminescence (Pierce; Thermo Fisher Scientific, Inc.) was performed. ImageJ software (version 1.47t; National Institutes of Health, Bethesda, MD, USA) was used to analyse the grey value of each target band, which indicated the expression level of the target protein compared with β-actin.

Wound-healing assay. The cells were seeded in a 6-well plate at 4.5×10^5 cells/well and allowed to grow to 90% confluence. The bottoms of the 6-well plates were marked with lines, and the wounds were scratched vertically with a 200 μL pipette tip. After washing 3 times with phosphate-buffered saline (PBS), the representative scrape lines for each set were photographed by a phase-contrast microscope at 0, 24 and 48 h. Shikonin at different concentrations was dissolved in DMEM with 1% FBS.

Immunofluorescence confocal microscopy. Cells were cultured on coverslips in 12-well plates for 24 h. After drug treatment, the cells were fixed with 4% paraformaldehyde for 20 min at room temperature, permeabilized with 0.3% Triton X-100 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; Proteintech, Wuhan, China) overnight at 4°C. Then, the cells were washed 3 times with ice-cold PBS, followed by incubation with the anti-rabbit secondary antibody for 60 min. Then, DAPI was used for nuclear staining. Finally, images were captured with a fluorescence microscope and quantified with Image-Pro Plus 6.0 software.

Colony formation assay. Two hundred cells per well were plated in 6-well plates and allowed to grow for approximately 5 to 7 days until small colonies could be clearly seen. Cells were treated for approximately

8 to 12 days with different concentrations (0, 2.5, and 3.5 μM) of Shikonin in DMEM growth media. Cells were fixed with 4% formaldehyde for 15 minutes. The colonies were stained with 0.2% crystal violet solution in 10% ethanol for 10 minutes. Excess stain was removed by washing repeatedly with PBS. All of the procedures were conducted at room temperature. Image-Pro Plus 6.0 software was used for quantification.

Reverse transcription-quantitative PCR (RT–qPCR). Briefly, total cellular RNA was extracted using TRIzol reagent. According to the manufacturer's instructions from Invitrogen (Thermo Fisher Scientific, Inc.), RT was performed to obtain cDNAs. The following are the PCR primers used for the analysis: MTA1, forward: 5'-CAT CAG AGG CCA ACC TTT TCG-3' and reverse: 5'-GCA CGT ATC TGT CGG TGG TC-3'; TGFβ1, forward: 5'-ACT CTC TGA CTT CCG CGT TC-3' and reverse: 5'-CAC TTG CCC AGC AAT AGG TTT AT- 3'; VEGF, forward: 5'-CTG GGC TGT TCT CGC TTC G-3' and reverse: 5'-CTC TCC TCT TCC TTC TCT TCT TCC-3' and GAPDH, forward: 5'-ACA ACT TTG GTA TCG TGG AAG AAG-3' and reverse: 5'-GCC ATC ACG CCA CAG TTT C-3'. The 20-μl PCR system contained 2 μl cDNA, 0.4 μl ROX Reference Dye II, 10 μl SYBR Premix Ex Taq II, 0.8 μl 10 μM sense primer, 0.8 μl 10 μM anti-sense primer and 6 μl ddH2O. The following were the conditions of the amplification process: 95°C for 30 s, 40 cycles of denaturation for 5 s at 95°C, 34 s of annealing at 60°C, elongation at 95°C for 15 s, and extension at 60°C for 1 min.

Statistical analysis. Where indicated, data are represented as the mean ± SEM and then were analysed by GraphPad Software Prism 7.0, and comparisons were made by one/two-way ANOVA with Bonferroni's multiple comparison tests (more than one group) or Student's t-test (two groups). A value of $p<0.05$ was considered significant. *P < 0.05, **P < 0.01, ***P < 0.001 vs. the control group.

Results

Shikonin noticeably reduces cell proliferation and clonogenicity in cervical cancer cells.

Shikonin at different concentrations was added to the HeLa and SiHa cervical cancer cell lines. After incubation for 48 hours, the effect of Shikonin on cell viability was assessed using a CCK-8 assay. Under the same treatment time, Shikonin inhibited the proliferation of HeLa and SiHa cells more obviously with an increasing drug concentration, and the inhibition rate of Shikonin on cell proliferation was approximately 50% when the concentration of Shikonin was 2.5 μM (Fig. 1a, b). To further examine the effects of different treatment times on the proliferation of HeLa and SiHa cells, Shikonin at concentrations of 0, 2.5 and 3.5 μM was used for 24, 48 and 72 h. With prolonged administration time, the inhibitory effect of Shikonin on cell proliferation became increasingly obvious (Fig. 1c, d). The above experimental results suggested that Shikonin can markedly inhibit the proliferation of cervical cancer cells in a dose- and time-dependent manner.

Ki67 is a nuclear antigen related to cell proliferation and it is an important indicator of tumour cell proliferation. The more Ki67-positive cells there are, the faster the cell proliferation rate. HeLa and SiHa cells were treated with 2.5 μM and 3.5 μM Shikonin for 48 h and then stained with Ki67 antibody (red) and DAPI (blue). The number of red fluorescence of Ki67 in the experimental group was significantly

decreased, and the fluorescence intensity was also significantly weakened. These results indicate that Shikonin has an obvious inhibitory effect on the proliferation of HeLa and SiHa cervical cancer cells (Fig. 1e-h).

Colony formation experiments are an effective method to reflect the proliferation ability of cells. The two kinds of cells were uniformly plated in six-well plates and treated with different concentrations of Shikonin. The experimental results showed that Shikonin significantly inhibited the size of HeLa and SiHa cervical cancer cell colonies. With increasing drug concentration, the space between cells became larger, and the shape of the clonogenic body became increasingly smaller (Fig. 1i-l).

Shikonin obviously reduces the phosphorylation levels of FAK, AKT and GSK3β in cervical cancer cells.

Focal adhesion kinase (FAK) is a key regulator of growth factor receptor and integrin-mediated signalling. It regulates the basic processes of cancer cell proliferation, migration, invasion and apoptosis through its kinase activity and scaffolding function[21]. Similarly, AKT (protein kinase B) and GSK3β (glycogen synthase kinase 3beta) also play a vital role in the growth of tumour cells[22, 23]. To evaluate whether FAK, AKT and GSK3β were involved, we measured the phosphorylation levels of FAK, AKT and GSK3β in cervical cancer cells after 2.5 μM Shikonin treatment. We found a significant decrease in FAK, AKT, and GSK3β phosphorylation but no changes in FAK, AKT, and GSK3β total protein expression (Fig. 2a-h). Shikonin may inhibit the proliferation of cervical cancer cells through FAK/AKT/GSK3β signalling.

Shikonin blocks the EGF-induced FAK/AKT signalling pathway in cervical cancer cells.

Next, we explored the effects of Shikonin on EGF-induced FAK, Akt and GSK3β phosphorylation. In the absence or presence of EGF (10 ng/ml), we treated cells with Shikonin. In cervical cancer cells, EGF upregulated the phosphorylation of FAK, AKT and GSK3β, while Shikonin significantly reduced EGF-induced FAK, AKT and GSK3β phosphorylation (Fig. 3a, b, e, f). Subsequently, to prove that AKT is downstream of FAK, we observed the effect of pf-562271, a small molecule selective inhibitor of FAK, on EGF-induced AKT phosphorylation in HeLa and SiHa cells. We pretreated cells with PF-562271 (10 μM) for 60 minutes and then treated the cells with EGF (10 ng/ml) for 60 minutes. We found that PF-562271 inhibited basal and EGF-induced AKT phosphorylation (Fig. 3c, d, g, h), which suggested that AKT could be a downstream factor of FAK. Therefore, we speculate that the inhibitory effect of Shikonin may be mediated through the FAK/Akt/GSK3β pathway.

Shikonin inhibits the EGFR-induced proliferation of cervical cancer cells through the FAK/AKT/GSK3β pathway.

To prove our hypothesis, we further detected whether Shikonin could inhibit the proliferation of cervical cancer cells induced by EGF. The cells were treated with Shikonin in the absence or presence of EGF. The results showed that EGF could significantly promote the proliferation of cervical cancer cells, while the proliferation-promoting effect of EGF could be significantly inhibited by Shikonin (Fig. 4a, b). Consistent with the western blot results, PF-562271 also dramatically inhibited this proliferative effect (Fig. 4c, d).

Generally, these results indicated that Shikonin could inhibit the proliferation of cervical cancer cells via the FAK/AKT/GSK3β signalling pathway.

Shikonin significantly inhibits cell migration and decreases the expression level of related proteins

Cell migration plays an important role in tumour progression, and Shikonin has a significant inhibitory effect on the proliferation, migration and invasion of various tumour cells. Therefore, we evaluated the effect of Shikonin on the metastatic ability of HeLa and SiHa cells using a wound healing assay. With the increase in drug concentration and the extension of treatment time, the inhibition rate of Shikonin on the migration of cervical cancer cells became more obvious (Fig. 5a-d). To further explore the molecular mechanism by which Shikonin affects tumorigenesis, we further detected the transcription level of genes related to cell metastasis and cycle regulation. Shikonin significantly inhibited the expression of MTA1, TGFβ1 and VEGF in HeLa cells (Fig. 5e).

Discussion

In recent years, several reports have shown that Shikonin has a significant inhibitory effect on the occurrence of many tumours[24], but its mechanism in cervical cancer has not been studied much. We found that Shikonin has a significant inhibitory effect on the proliferation of HeLa and SiHa cervical cancer cells, and this effect may be achieved by regulating the phosphorylation of the FAK/AKT/GSK3β pathway. In addition, Shikonin can significantly inhibit the migration of cervical cancer cells and reduce the transcriptional levels of the MTA1, TGFβ1 and VEGF genes.

Shikonin has been reported to have a significant inhibitory effect on a variety of tumours in vitro and in vivo. Tang et al. found that Shikonin inhibited the proliferation of oesophageal cancer cells both in vitro and in vivo and may be associated with the induction of apoptosis and cell cycle arrest through inhibition of the HIF1α/PKM signalling pathway[25]. Shikonin can also inhibit the proliferation and induce apoptosis of human leukaemia NB4 cells by regulating MAPKs and C-MyC[26]. Interestingly, Shikonin can block EMT, migration and invasion of human lung adenocarcinoma cells in the inflammatory microenvironment through the IL-6/STAT3 signalling pathway[27]. In the present study, we performed CCK8 assays on two representative strains of HeLa and SiHa cervical cancer cells following the use of Shikonin. The results showed that the cell viability decreased after treatment with Shikonin at 0.0-4.0 μM. Then, Shikonin at 0, 1.5, 2.5 and 3.5 μM was used for 24 h, 48 h and 72 h, and the inhibitory effect of Shikonin on cell viability became increasingly obvious as the administration time increased. The above results showed that Shikonin significantly inhibited the proliferation of cervical cancer cells in a dose-dependent and time-dependent manner. Ki67 is a nuclear antigen associated with the cell cycle and is regarded as one of the most reliable indicators of proliferative activity of tumour cells. Using immunofluorescence, we found that the number and intensity of Ki67 fluorescence was significantly reduced in HeLa and SiHa cells after Shikonin treatment. In addition, the experimental results of clonal colony formation assay showed that the number of colonies was significantly reduced after Shikonin

treatment, the gaps between cells became larger and the sizes of clone-forming bodies became smaller as the administration concentration increased. In conclusion, Shikonin has a significant inhibitory effect on the proliferation of cervical cancer HeLa and SiHa cells.

FAK is an important regulatory molecule in growth factor receptor and integral protein-mediated signalling pathways, it usually plays a role in a kinase-dependent manner[28]. In tumours of multiple tissue origins, the expression of activated FAK is increased significantly[29]. FAK is commonly overexpressed or hyperphosphorylated in tumour cells and it promotes glucose consumption, adipogenesis and glutamine dependence to promote cancer cell proliferation, motility and survival[30]. AKT is a serine/threonine protein kinase that plays an important role in cell survival and apoptosis, and studies have shown that AKT activation can affect the tumour cell growth, proliferation, migration, apoptosis and tumour angiogenesis of tumour cells by regulating many downstream proteins.[31] Several studies have shown that the PI3K/AKT/GSK3β signalling pathway regulates epithelial mesenchymal transition in many tumours, thus participating in tumour invasion and metastasis[32–34]. However, the FAK/AKT/GSK3β signalling pathway has not been studied much in cervical carcinogenesis. In our study, we found that Shikonin significantly inhibited the phosphorylation levels of FAK, AKT and GSK3β proteins in cervical cancer cells.

To investigate in depth the mechanism by which Shikonin inhibits the proliferation of cervical cancer cells, we first detected the effect of Shikonin on the EGFR-induced signalling pathway. EGFR is an important tyrosine kinase receptor in tumorigenesis and it plays an important role in tumour cell proliferation, migration and apoptosis[35]. Phosphorylation levels of FAK and AKT were significantly increased when cells were stimulated with EGF alone, whereas cotreatment with Shikonin resulted in a significant decrease in EGF-mediated intracellular FAK and AKT phosphorylation, indicating that Shikonin can inhibit the phosphorylation of FAK and AKT mediated by EGF.

We found that 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 is the downstream signalling molecule of FAK. In addition, we treated cervical cancer cells with EGF and PF-562271 alone or together. The western blot results showed that PF-562271 inhibited the EGF-induced increase in FAK and AKT phosphorylation levels, and the CCK-8 assay also showed that PF-562271 significantly inhibited EGF-induced cell proliferation. We speculate that this is because inhibition of FAK phosphorylation causes a decrease in downstream AKT phosphorylation, which in turn weakens the proliferation of cervical cancer cells. In conclusion, we found that the inhibition of cervical carcinogenesis by Shikonin may be mediated through the FAK/AKT/GSK3β phosphorylation signalling pathway.

Meanwhile, the wound-healing assay showed that the cell migration ability decreased after Shikonin treatment. Furthermore, we found that Shikonin could inhibit the expression of MTA1, TGFβ1 and VEGF, which suggested that Shikonin may inhibit the proliferation of tumour cells by affecting downstream related cyclins after inhibiting the phosphorylation of FAK and AKT, which also provided us with potential ideas for subsequent studies. In addition, in the future, we can continue to validate the tumour

suppressive effect of Shikonin in mice with subcutaneous tumours or in situ cervical cancer in vivo, and we can also take tumour tissues from animals for protein, RNA and immunohistochemical staining to detect the expression of p-FAK, p-AKT and Ki67 proteins to better elaborate the molecular mechanisms involved.

In conclusion, we found that Shikonin could significantly inhibit the proliferation and migration of cervical cancer cells. We also clarified that Shikonin inhibited the proliferation of cervical cancer cells, possibly by regulating the EFG-mediated FAK/AKT/GSK3β phosphorylation signalling pathway. Our findings suggest that Shikonin may be a potential drug for the clinical treatment of cervical cancer, and our research also provides some theoretical basis for the development of new therapeutic drugs in the clinical setting.

Declarations

- **Ethics approval**
  The study was approved by the appropriate institutional research ethics committees.
- **Consent to participate**
  Not applicable.
- **Consent for publication**
  Not applicable.
- **Availability of data and materials**
  The datas and materials used during the study are available.
- **Competing interests**
  All authors declare that they have no competing interest.
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- **Authors' contributions**
  ZX and LH conducted the experiments. TZ, YL, MG and XW analyzed most of the data. WC, LL and YZ analyzed the western blotting data. NL and PH conceived and designed the study. ZX and PH wrote the manuscript. All authors read and approved the final manuscript.
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- **Compliance with Ethical Standards**
- **Ethics approval and consent to participate**
  Ethics approval for the study was obtained from Nanchang University Institutional Research Ethics Committee.The procedures used in this study adhere to the tenets of the Declaration of Helsinki.
- **Disclosure of potential conflict of interests**
  All authors declare that they have no competing interest.
- **Research involving human participants and/or animals**
  Not applicable.
- **Informed consent**
  Not applicable.

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Figures

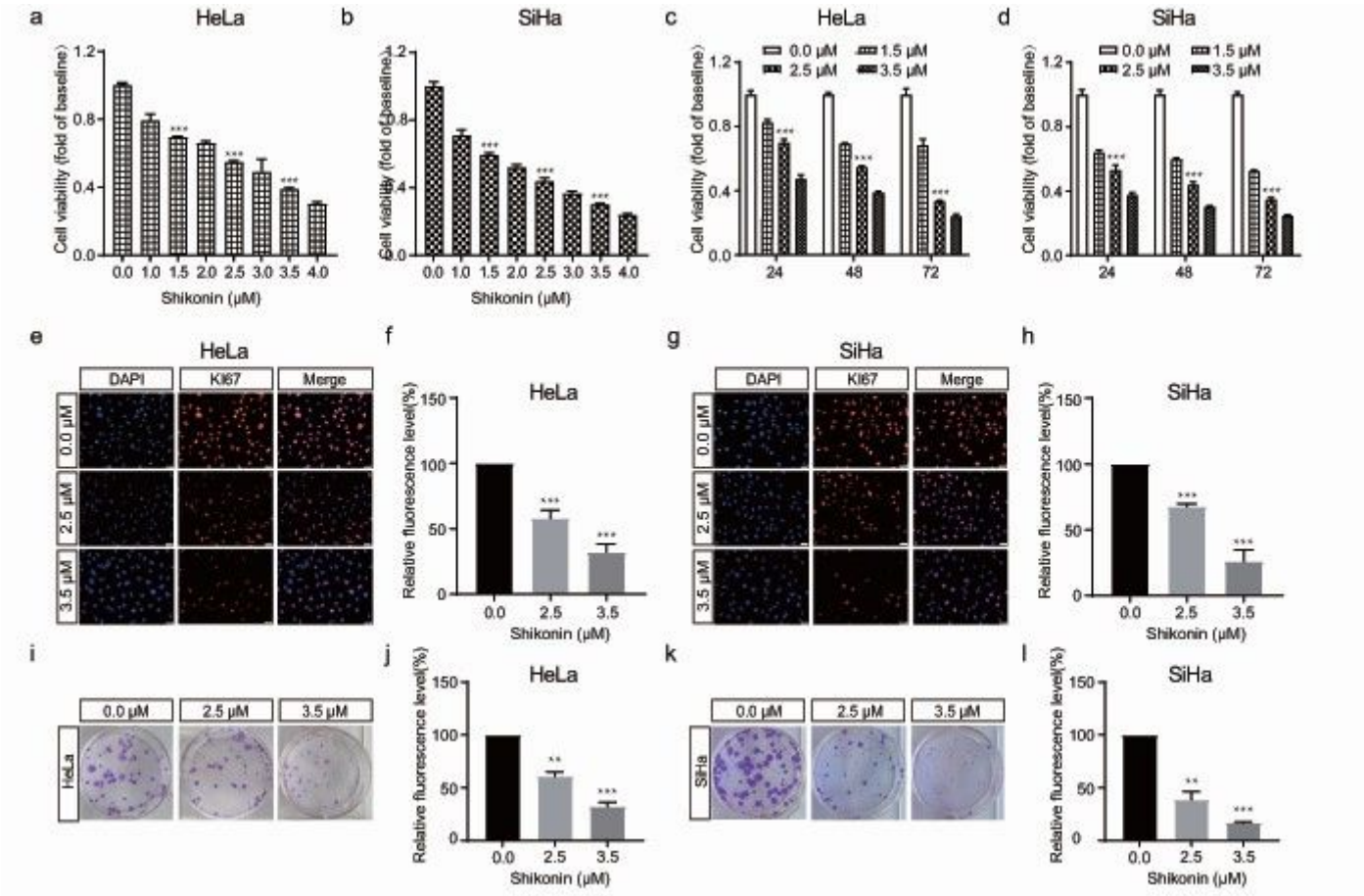

Figure 1

Shikonin noticeably reduces cell proliferation and clonogenicity in cervical cancer cells.

(a, b) Dose-dependent effects of Shikonin on the proliferation of cervical cancer cells. HeLa (a) and SiHa (b) cells were treated with increasing concentrations of Shikonin as indicated above for 48 h. (c, d) Time-dependent effects of Shikonin on the proliferation of cervical cancer cells. HeLa (c) and SiHa (d) cells were treated with Shikonin for 24, 48 and 72 h. (e, g) Immunofluorescence confocal microscopy was used to assess the expression of Ki67 in cervical cancer cells after Shikonin treatment. Scale bar, 50 μm, 20×objective. (f, h) Quantitation of the fluorescence level as shown in e&g. (i, k) Colony formation experiment of SiHa and HeLa cells after incubation with Shikonin at different concentrations. (j, l) Quantitation of the colony formation results shown in i&k.

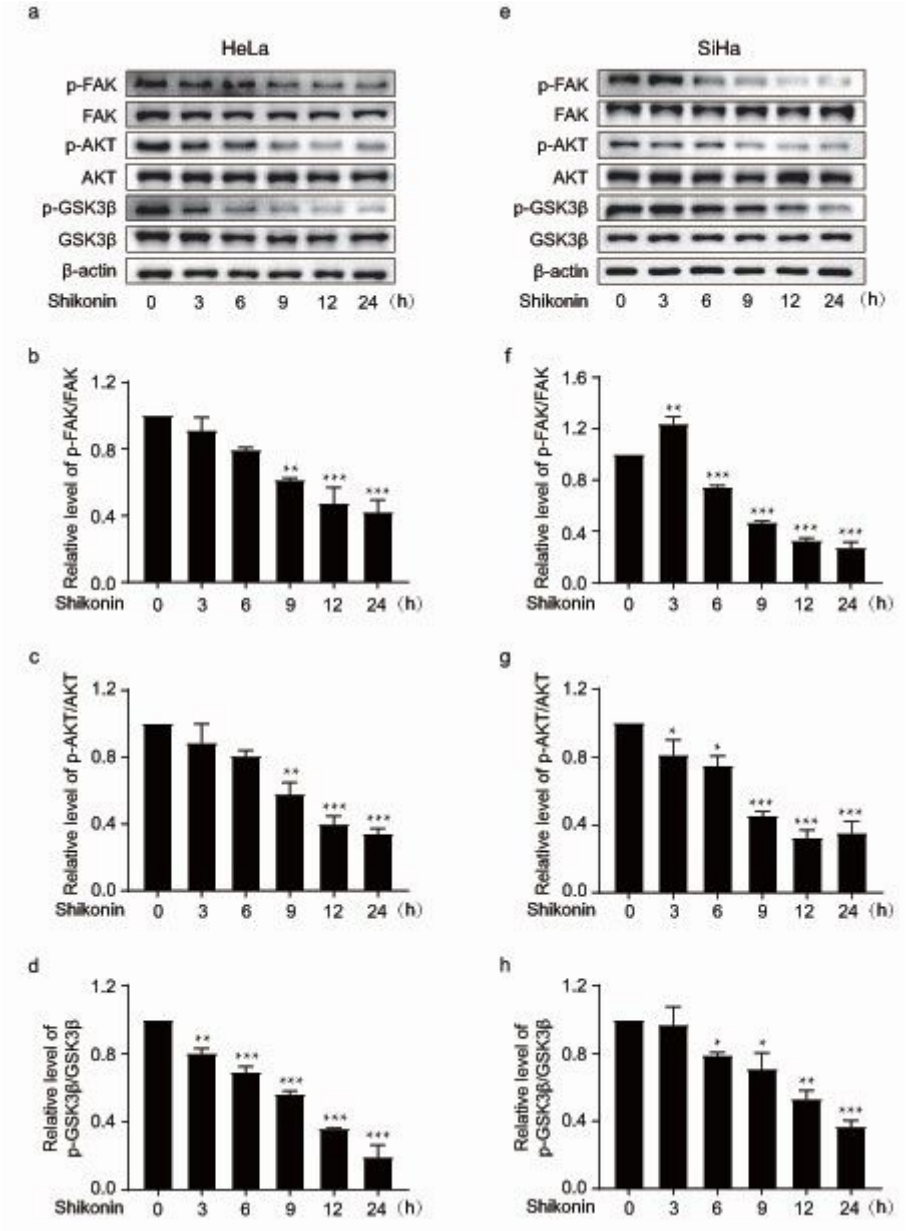

Figure 2

Shikonin obviously reduces the phosphorylation levels of FAK, AKT and GSK3β in cervical cancer cells.

Phosphorylation levels of FAK, AKT, and GSK3β after 2.5 μM Shikonin treatment at different times in HeLa (a) and SiHa (e) cells, as measured by western blot analysis. (b-d, f-h) Quantitation of the western blot results.

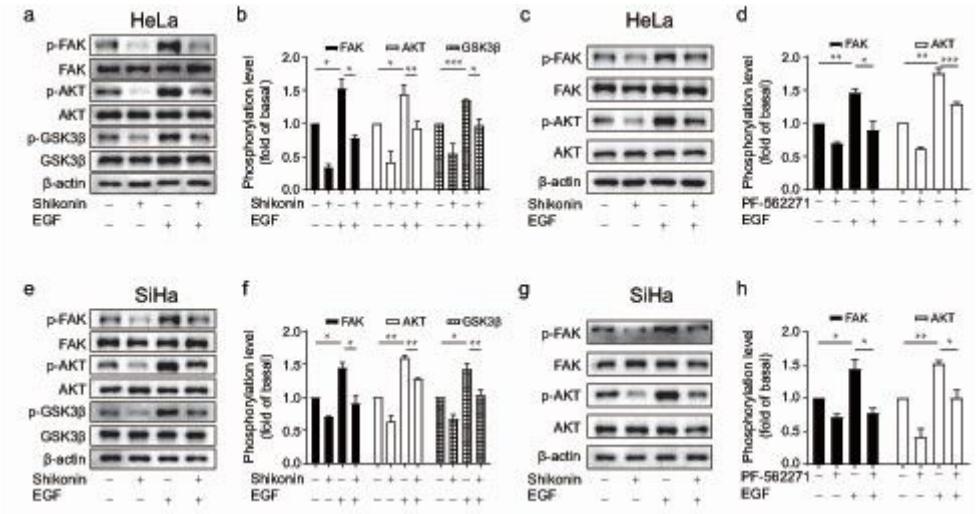

Figure 3

Shikonin blocks the EGF-induced FAK/AKT signalling pathway in cervical cancer cells.

(a, e) Effects of Shikonin on EGF-induced FAK and AKT phosphorylation in HeLa and SiHa cells. (b, f) Quantitation of western blot results. (c, g) Effects of PF-562271 on EGF-induced FAK and AKT phosphorylation in HeLa and SiHa cells. (d, h) Quantitation of western blot results.

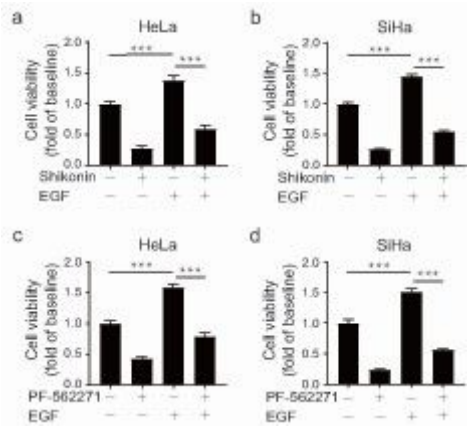

Figure 4

Shikonin inhibits the EGFR-induced proliferation of cervical cancer cells through the FAK/AKT/GSK3β pathway.

(a, 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 measured by CCK-8 assay. (c, 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.

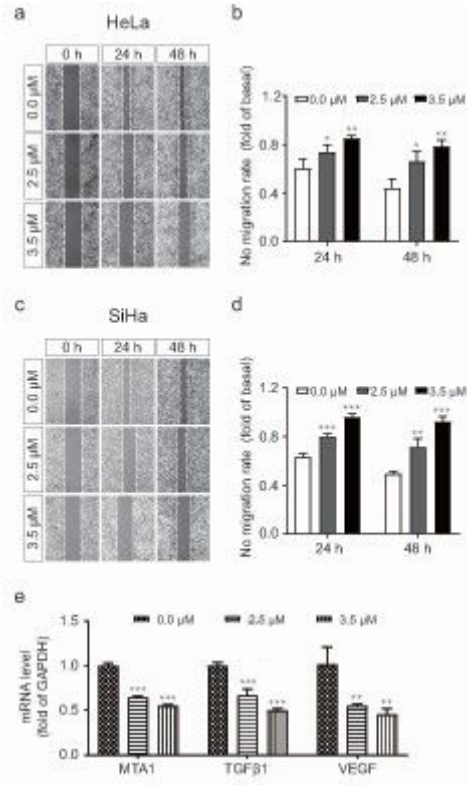

Figure 5

Shikonin significantly inhibits cell migration and decreases the expression level of related proteins.

(a, 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 wound healing assay. (b, d) The relative no migration rate was calculated by measuring the width of at least three wounds and normalized to the control. (e) Effects of Shikonin on MTA1, TGFβ1 and VEGF in HeLa cells.