# Downregulation of Krüppel-like factor 1 inhibits the metastasis and invasion of cervical cancer cells

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**Abstract.** Cervical cancer is one of the most common malignancies that seriously threatens women's health. Krüppel-like factors (KLFs) have been reported to be associated with the progression of cervical cancer. The role of KLF1 in cervical cancer, which still remains unclear, was investigated in the present study. The expression of KLF1 was detected in different cervical cell lines by reverse transcription-quantitative polymerase chain reaction (RT-qPCR) and western blotting. Cell proliferation, metastasis and invasion were respectively detected by Cell Counting Kit-8, wound healing and transwell assays. Associated factor expression was also detected by RT-qPCR and western blotting. In addition, the phosphorylation levels of phosphatidylinositol-3-kinase (PI3K) and protein kinase B (Akt) were determined by western blot analysis. The results revealed that KLF1 expression was promoted in SiHa, Caski and C4-1 cervical cancer cells. However, KLF1 knockdown suppressed cell proliferation, metastasis and invasion in SiHa cervical cancer cells. KLF1 knockdown also inhibited the expressions of Ki67, metastasis-associated antigen 1 and matrix metalloproteinase (MMP)-2. KLF1 knockdown promoted the expressions of nonmetastatic clone 23 type 1 and tissue inhibitor of metalloproteinase-2, and the expression of MMP-9 was promoted slightly as well. In addition, KLF1 knockdown inhibited the PI3K/Akt signaling pathway. Hence, it was concluded that KLF1 promoted metastasis and invasion via the PI3K/Akt signaling pathway in cervical cancer cells.

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## Introduction

Cervical cancer, a malignant tumor that occurs in the cervical epithelium, is one of the most common malignancies that seriously threatens women's health worldwide (1). It is regarded as the most serious malignancy in women reproductive system (2,3), with the annual number of newly diagnosed patients and deaths being approximately 529,800 and 275,100 cases. Noticeably, over 85% of cervical cancer patients and deaths cause by it are in developing countries (4). The 5-year survival rates of patients who have with early-onset cervical cancer and without pelvic lymph node metastasis ranges from 85 to 90%. Once regional lymph node metastases occurs, the 5-year survival rates drop to 30 to 60% (5). However, according to the cancer's pathophysiological characteristics, cervical cancer is prone to re-occurrence and metastasize 10-15% of patients with cervical cancer still show recurrence and metastasis even if the pathological examination shows no sign of lymph node metastasis (6). Some cervical cancer tumor cells is strongly invasive, which would seriously affect the prognosis of patients (7).

It has been reported that the incidence of cervical cancer was associated with abnormal expression of some related molecules including inactivation of various tumor suppressor genes and activation of oncogenes (8). Previous studies have found that transcription factors played important roles in the development and progression of many types of cancers. The activated signal transduction pathways convert extracellular signals into intranuclear signals, and the activation of transcription factors leads to the expression or suppression of downstream genes. These two process eventually changed carcinogenicity. Under both normal development and disease conditions, Krüppel-like factors (KLFs) play roles in the DNA transcription mechanisms, and participate in many biological processes such as cell proliferation (9,10), differentiation (11), migration (12) and apoptosis (13). The KLF gene was first discovered in the developmental regulation factor Krüppel in *Drosophila* embryos (14). The first mammalian KLF gene, KLF1 or EKLF, was discovered in 1993 (15), followed different homologous genes being subsequently discovered (16). To date, KLF3 (17), KLF4 (18) and KLF5 (19) have been reported to be associated with the occurrence, progression and prognosis of cervical cancer. However, the role of KLF1 in cervical cancer still remains unclear.

Understanding the molecular mechanisms of the occurrence, development, recurrence and metastasis of cervical cancer is of significance. Such an understanding help guide more in-depth studies on early diagnosis and conduct individualized treatment of cervical cancer. Therefore, we determined to study the role and molecular mechanism of KLF1 on cervical cancer.

## Materials and methods

*The cBioPortal survival analysis*. The 2-year survival analysis in the cervical cancer patients with or without alterations in query genes of KLF1, was obtained from online analysis of cBioPortal website.

*Cell culture*. Human cervical cancer cell lines, SiHa (contains a complete HPV16 genome), Caski (HPV16+, contains approximately 400 copies of the HPV16 genome) and C4-1 (HPV18+, contains a complete HPV18 genome) and normal cervical cells were acquired from American Type Culture Collection (ATCC; Guangzhou, China). The normal cervical epithelial cells (HcerEpic) were purchased from BeNa Culture Collection (Jiangsu, China). Cells were cultured in Dulbecco's modified Eagle's medium (DMEM, high glucose) (HyClone; GE Healthcare Life Sciences, Logan, UT, USA) with 10% (v/v) fetal bovine serum (FBS; Biological Industries, Kibbutz Beit-Haemek, Israel) and 100 U/ml streptomycin and 100 $\mu$g/ml penicillin (Biological Industries, Israel) in an incubator with 5% $CO_2$ at 37˚C. Cells were grown on plastic dishes and prepared for mRNA and protein extraction.

*siRNA interference*. The sequence of small interfering RNA (siRNA) for KLF1 and non-specific sequence (control siRNA for Mock group) were synthesized by Ribobio (Guangzhou, China). Using Lipofectamine 3000 transfection reagent (Invitrogen, USA), SiHa cells were transfected with 1 $\mu$g siRNA after the cell confluence reached 70%.

*Cell viability detection*. Cell Counting Kit-8 (CCK-8; Beyotime Institute of Biotechnology, Haimen, China) was applied to determine cell viabilities. To be more specific, cells ($5x10^3$ /well) were inoculated in 96-well plates. After being incubated for 24 h (h), cells were stained with 20 $\mu$l staining reagent for 1 h. The optical density (OD) values at 450 nm were measured by 1500 microplate reader (Thermo Fisher Scientific, Inc., Waltham, MA, USA).

*Wound healing assay*. Wound healing assay was used to determine cell metastasis abilities. To be more specific, $1x10^5$ cells were inoculated in each well of 12-well plates and incubated at 37˚C for 24 h. Next, the confluent monolayer cells were first scratched gently to form a cell-free area and then cultured in an incubator at 37˚C for 24 h. Finally, the diameters of cell-free areas were measured under Olympus DSX100 optical microscope (Olympus Corporation, Tokyo, Japan).

*Transwell assay*. Using 24-well Transwell chambers containing 8-$\mu$m pore filters (Corning Incorporated, Corning, NY, USA), cell invasion abilities of cervical cancer cells with KLF1 interference were compared to Control and Mock groups. To explain, $5x10^4$ cells were cultured in Matrigel GFR (BD Biosciences, Franklin Lakes, NJ, USA)-coated Transwell upper chambers using DMEM culture media. Meanwhile, DMEM culture media containing 10% FBS was filled in the lower chambers. After the incubation at 37˚C for 24 h, the bottom membrane was stained with 0.1% crystal violet at 37˚C for 30 min. Cell numbers was calculated using Olympus DSX100 optical microscope (Olympus Corporation) with the magnification set at 100-fold.

*Reverse transcription-quantitative polymerase chain reaction (RT-qPCR)*. The mRNA expression levels of KLF1, apoptosis and metastasis related factors were determined by RT-qPCR. Total RNA was extracted from cells with TransZol Up (Beijing Transgen Biotech Co., Ltd., Beijing, China), and cDNA was reverse transcribed by cDNA synthesis SuperMix kit (Beijing Transgen Biotech Co., Ltd.). RT-qPCR amplification was carried out by using LightCycler® SYBR Green I Masters (Roche Diagnostics, Indianapolis, IN, USA) in a LightCycler® 480II System (Roche Diagnostics), and the conditions were set as follows: Initial denaturation at 95˚C for 5 min (min), 40 cycles (denaturation at 95˚C for 15 sec, annealing at 57˚C for 15 sec, extension at 72˚C for 25 sec) and final extension at 72˚C for 10 min. The primer sequences of KLF1, Ki67, nonmetastatic clone 23 type 1 (Nm23-H1), metastasis-associated antigen (MTA-1), matrix metalloproteinase (MMP)-2, MMP-9 and tissue inhibitor of metalloproteinase-2 (TIMP-2) are listed in Table I. Gene expression was quantified according to the $2^{-\Delta\Delta Cq}$ method (20).

*Western blot analysis*. Cells were rinsed by phosphate-buffer saline (PBS), lysed by Protein Lysis Reagent P0013 (Beyotime, China) for 20 min and centrifuged on ice at 12,000 x g for 10 min. The supernatant with proteins was first harvested and then quantified by using BCA protein assay reagent (Beyotime Institute of Biotechnology). Subsequently, total proteins (20 $\mu$g/lane) were subjected to 15% sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) and then separated. Next, the gel was transferred onto a piece of polyvinylidene fluoride (PVDF) membranes (Thermo Fisher Scientific, Inc.). The membranes were first blocked using 5% non-fat dry milk for 1 h at 37˚C and then incubated with specific primary antibodies overnight at 4˚C: Rabbit anti-KLF1 (ab49158, 1:400; Abcam, Cambridge, UK), Ki67 (ab92742, 1:1,000; Abcam), nm23-H1 (ab154547, 1:2,000; Abcam), MTA-1 (ab71153, 1:5,000; Abcam), MMP2 (ab92536, 1:1,000; Abcam), MMP9 (ab38898, 1:1,000; Abcam), TIMP-2 (ab180630, 1:1,000; Abcam), phosphatidylinositol-3-kinase (PI3K) (ab133595, 1:2,000; Abcam), p-PI3K (ab182651, 1:1,000; Abcam), protein kinase B (PKB or Akt) (ab8805, 1:500; Abcam), p-Akt (ab38449, 1:1,000; Abcam) and GAPDH (ab9485, 1:2,000; Abcam). Subsequently, the membranes were probed with Goat Anti-Rabbit IgG H&L (HRP) secondary antibody (ab6721, 1:5,000; Abcam) for 1 h at 37˚C. The immunoblots were visualized by GE ECL (enhanced chemiluminescence) Start Detection reagents (GE Healthcare Life Sciences, Little Chalfont, UK). Digital images of immunoreactive bands were obtained by a Bio-Rad ChemiDoc XRS densitometry with Image Lab™ Software no. 1708265 (Bio-Rad Laboratories, Inc., Hercules, CA, USA).

Table I. Primer sequences applied in the present study.

| Name | Type | Sequence (5'-3') |
|---|---|---|
| GAPDH | Forward | CCATCTTCCAGGAGCGAGAT |
| | Reverse | TGCTGATGATCTTGAGGCTG |
| KLF1 | Forward | GAAGAGGACGATGAGAGGGG |
| | Reverse | ATCCTCCGAACCCAAAAGC |
| Ki67 | Forward | TCAAAAGGAGTGGCGGAGAT |
| | Reverse | AAAACCTCCCCATTGACACG |
| nm23-H1 | Forward | GGGCTGAATGTGGTGAAGAC |
| | Reverse | GAAACCACAAGCCGATCTCC |
| MTA-1 | Forward | CTACGACCCACAGCAGAAGA |
| | Reverse | TGGTCGATCTGCTTGTCTGT |
| MMP-2 | Forward | CAGCCCTGCAAGTTTCCATT |
| | Reverse | GTTGCCCAGGAAAGTGAAGG |
| MMP-9 | Forward | GAGACTCTACACCCAGGACG |
| | Reverse | GAAAGTGAAGGGGAAGACGC |
| TIMP-2 | Forward | TTCAAAGGGCCTGAGAAGGA |
| | Reverse | TCAGGCTCTTCTTCTGGGTG |

KLF1, Krüppel-like factor 1; nm23-H1, nonmetastatic clone 23 type 1; MTA, metastasis-associated antigen; MMP, matrix metalloproteinases; TIMP, tissue inhibitor of metalloproteinase-2.

*Statistical analysis*. Statistical analysis was carried out using SPSS 22.0 (IBM Corp., Armonk, NY, USA). All data were presented as the mean ± standard deviation. At least three repeated experiments were performed in each group. Significance differences were analyzed by one-way analysis of variance with Dunnett's post hoc test. The Kaplan Meier survival analysis with a log-rank test was used to draw the survival curve. $P<0.05$ was considered to indicate a statistically significant difference.

## Results

*The expression levels of KLF1 in cervical cancer patients and cell lines*. According to the online analysis of the cBioPortal website, there was no significant difference on the survival curves between cervical cancer cases with or without alterations in query genes ($P=0.392$) in 2 years. However, it could be seen from the figure that the survival rate was increased when KLF1 was up-regulated. Note that the results of this analysis were only for reference as discrepancies may exist from the experimental results (Fig. 1A).

The mRNA and protein levels of KLF1 in different cervical cancer cell lines were compared with normal cervical epithelial cells using RT-qPCR and western blot assays. The results demonstrated that both the mRNA and protein expression levels of KLF1 increased significantly in the 3 cervical cancer cell lines (SiHa, Caski and C4-1 cells), compared with those in normal cervical epithelial cells ($P<0.01$; Fig. 1B and C). Among all detected cells, KLF1 in SiHa cell lines had the expression levels among others. Such a results guided us to perform following KLF1-silencing experiments on SiHa cells.

*KLF1 interference inhibited cell proliferation, metastasis and invasion abilities of cervical cancer cells*. The transfection effect of KLF1-siRNA in SiHa cervical cancer cells was evaluated by RT-qPCR and western blot assays. The results demonstrated that both mRNA and protein levels of KLF1 decreased remarkably in KLF1-siRNA group, compared with Control and Mock groups ($P<0.01$; Fig. 2).

To investigate the effect of KLF1 on cervical cancer cell proliferation, we detected cell proliferation abilities using CCK8 assay. The cell proliferation abilities were inhibited in KLF1-siRNA group in a time-dependent (12, 24 and 48 h) manner, with significant differences shown at 24 and 48 h ($P<0.05$; Fig. 3A). The effect of KLF1 on cell metastasis and invasion abilities of cervical cancer cells was determined by using wound healing and transwell assays. The wound healing and invasion rates were inhibited significantly when KLF1 was knocked down in SiHa cervical cancer cells, compared with Control and Mock groups ($P<0.05$; Fig. 3B-E).

*KLF1 interference influenced the expression levels of cell proliferation and metastasis-related factors*. The expression levels of cell proliferation and metastasis-related factors such as Ki67, Nm23-H1, MTA-1, MMP-2, MMP-9 and TIMP-2 were respectively detected by RT-qPCR and western blot assays. The results showed that both the mRNA and protein expression levels of Ki67 decreased significantly when KLF1 was silenced. However, the mRNA and protein expression levels of Nm23-H1 was increased significantly ($P<0.01$; Fig. 4A, B and E). In addition, while the mRNA and protein levels of both MTA-1, MMP-2 decreased significantly when KLF1 was silenced, those of MMP-9 was decreased slightly, and those of TIMP-2 was increased significantly ($P<0.05$; Fig. 4C-E).

*KLF1 interference inhibited the activation of PI3K/Akt signaling pathway*. To understand the functional mechanism of KLF1 on cell proliferation, metastasis and invasion abilities of cervical cancer cells, we studied the relation between KLF1 and PI3K/Akt signaling pathway. We observed that the phosphorylation levels of PI3K and Akt proteins declined remarkably in KLF1-siRNA group, compared with Control and Mock groups ($P<0.01$; Fig. 5).

## Discussion

In many developing countries, the early detection strategies for cancer still face many problems. Cervical cancer is still relatively rampant is one of the leading causes of death among women in developing countries (21). Approximately 90% of cancer deaths are caused by metastases (22), such a figure makes tumor metastasis a critical cause of deaths among cancer patients.

KLFs take part in cell cycle, differentiation, tumor formation. Particularly, in cancers, KLFs function to enhance or inhibit the cell cycle of many types of cells, (19). In this research, we studied whether KLF1 functioned in cervical cancer cells. Although the data from cBioPortal online analysis showed no significant difference in terms of the survival curves between cervical cancer cases with and without alterations in query genes within 2 years, a previous

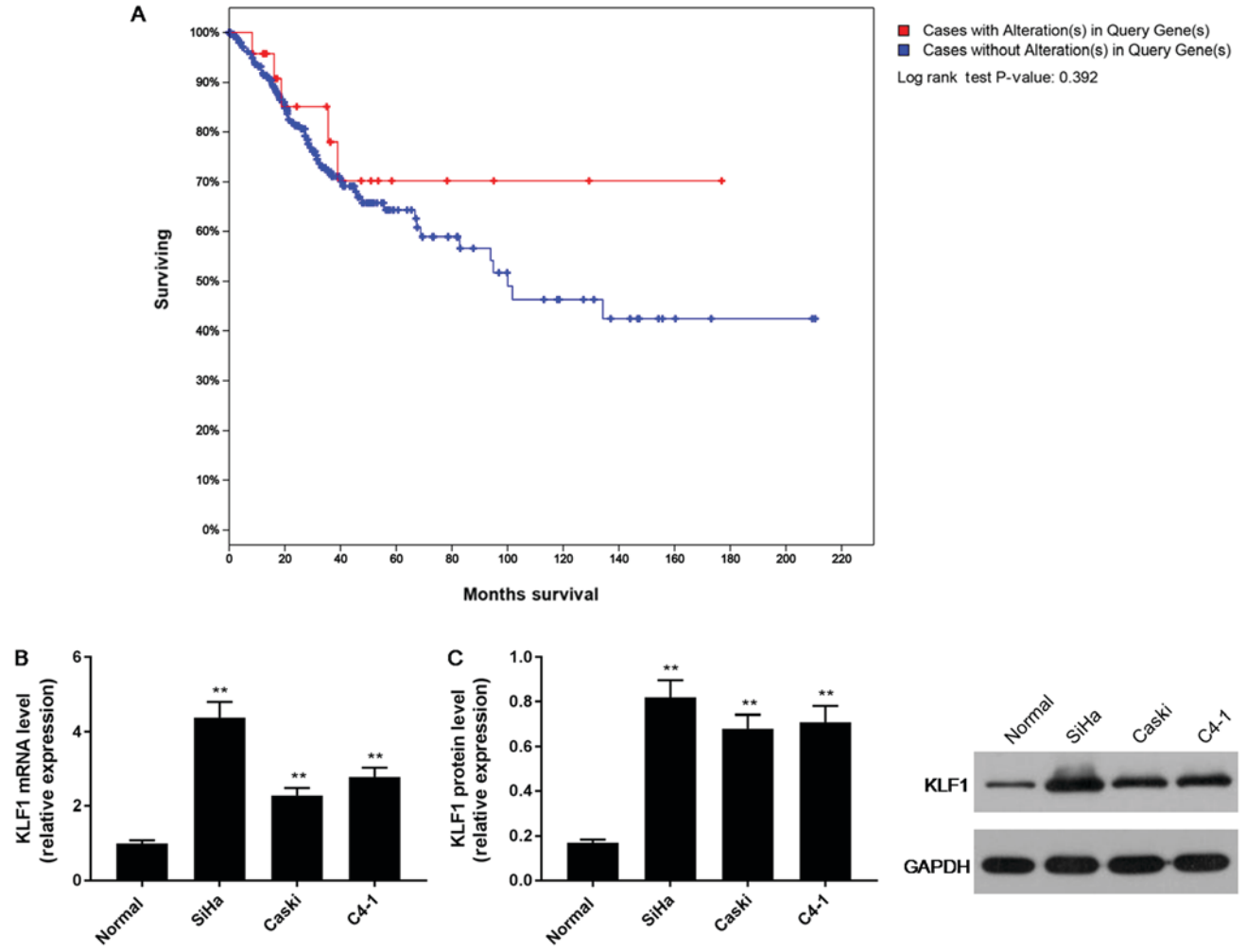


Figure 1. Expression levels of KLF1 in cervical cancer patients and cell lines. (A) The online survival analyses of cervical cancer cases, with and without alterations in query genes, on cBioPortal website. (B) The mRNA levels of KLF1 increased significantly in cervical cancer cell lines (SiHa, Caski and C4-1). (C) The protein levels of KLF1 increased significantly in cervical cancer cell lines (SiHa, Caski and C4-1). **P<0.01 vs. normal cells. KLF1, Krüppel-like factor 1.

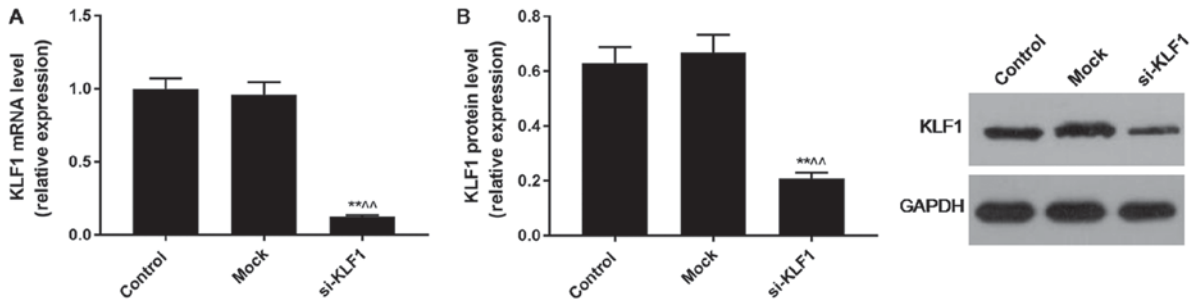


Figure 2. Transfection effect of KLF1-siRNA in SiHa cervical cancer cells. The (A) mRNA and (B) protein levels of KLF1 decreased significantly in the KLF1-siRNA group. **P<0.01 vs. Control group; ^^P<0.01 vs. Mock group. KLF1, Krüppel-like factor 1; si-/siRNA, small interfering RNA.

study documented that KLF1 mRNA expression was elevated cervical cancer tissues (19). Moreover, we found that KLF1 increased significantly in cervical cancer cells such as SiHa, Caski and C4-1 cells, particularly, in SiHa cells. The small size enrolled in the survival curves (cases with alteration, 20; cases without alteration, 284) may lead to the difference between survival curves and our results. In this study, cell viability, metastasis and invasion abilities were inhibited significantly when KLF1 was silenced in SiHa cells.

The expression of Ki67 have been reported to be directly correlated with the severity of cervical lesions (23,24). Essentially, Ki67 is an nuclear antigen, precisely, it is a non-histone protein located in the nucleus (25). As an important indicator pointing to the activity of tumor cells, Ki67 is actively expressed at each stage of cell division. Consistently, we found that Ki67 actively expressed in cervical cancer cells, and that the expression of Ki67 was down-regulated when KLF1 was knocked down.

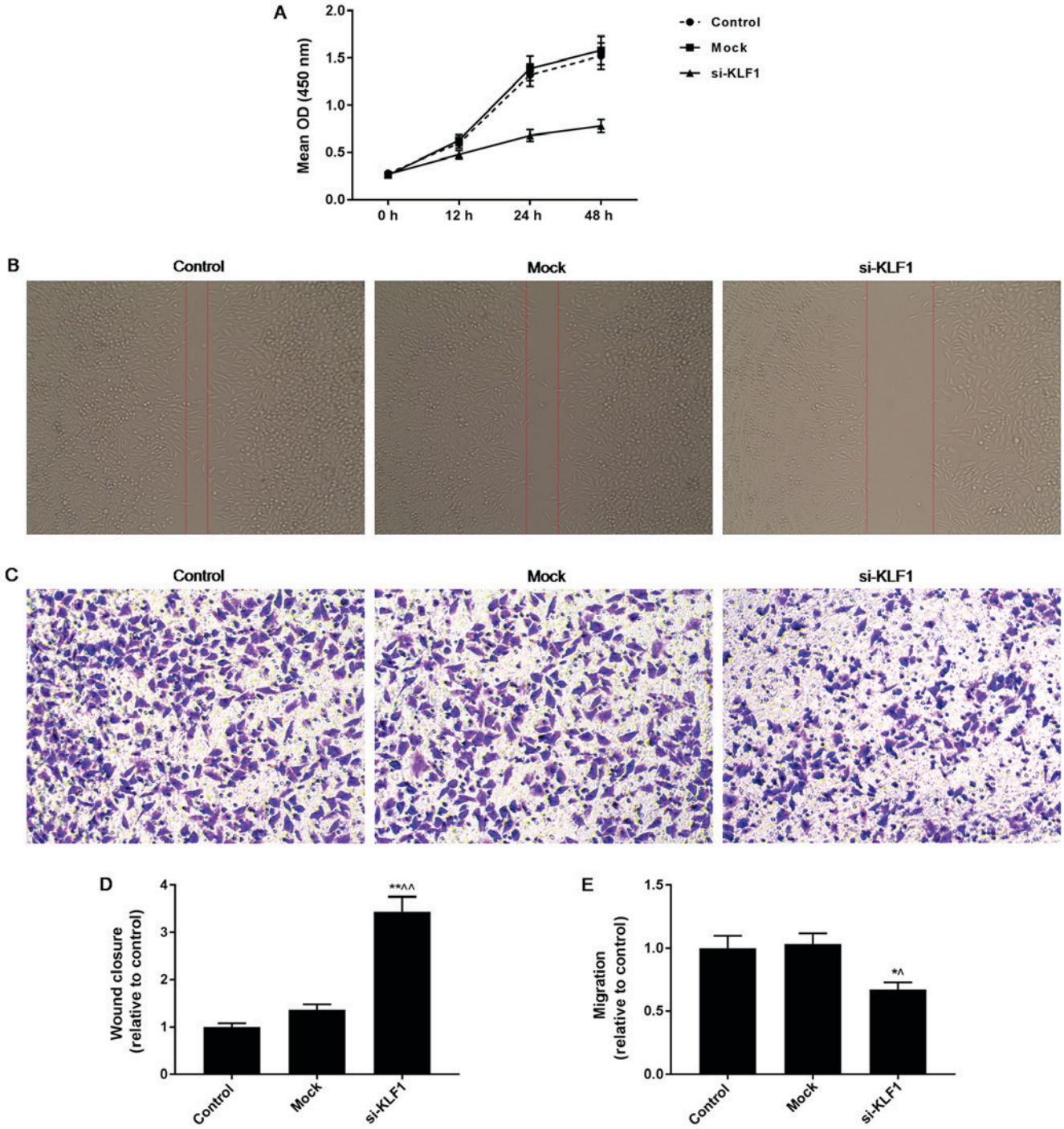


Figure 3. KLF1 interference inhibits the cell proliferation, metastasis and invasion abilities of cervical cancer cells. (A) Cell proliferation abilities were inhibited in the KLF1-siRNA group in a time-dependent manner (12, 24 and 48 h). (B and D) Using wound healing assays, the cell metastasis ability of cervical cancer cells was revealed to be inhibited in the KLF1-siRNA group. (C and E) The cell invasion ability of cervical cancer cells was inhibited, as determined by a transwell assay. Magnification, x100. $^{*}$P<0.05 and $^{**}$P<0.01 vs. Control group; $^{\wedge}$P<0.05 and $^{\wedge\wedge}$P<0.01 vs. Mock group. KLF1, Krüppel-like factor 1; si-/siRNA, small interfering RNA; OD, optical density.

Tumor metastasis is a complex process in which multiple pathways, steps and molecules are involved. Finding and identifying key regulatory molecules in this process is of great significance in the prevention and treatment of tumors in clinic. Nm23-H1 has diverse functions in the metastasis of many cancers which including cervical cancer (26). Nm23-H1 knockdown has been reported to be able to promote (27,28) or inhibit (29) cell invasion of cervical cancer. In our study, KLF1 knockdown promoted Nm23-H1 expression. MMPs could degrade extracellular matrix of invaded tissues and is critical for tumor metastasis (30-32). Our research showed that KLF1 knockdown inhibited MMP-2 expression. Nevertheless, the expression of MMP-9 remained stable under the interference of KLF1 knockdown. Some other signal molecules (not identified in this study) may also contribute to the regulation the progression of cervical cancer. In addition, our results showed that metastasis-associated antigen MTA was promoted, and that TIMP-2, the specific inhibitor of MMPs (33-35), was

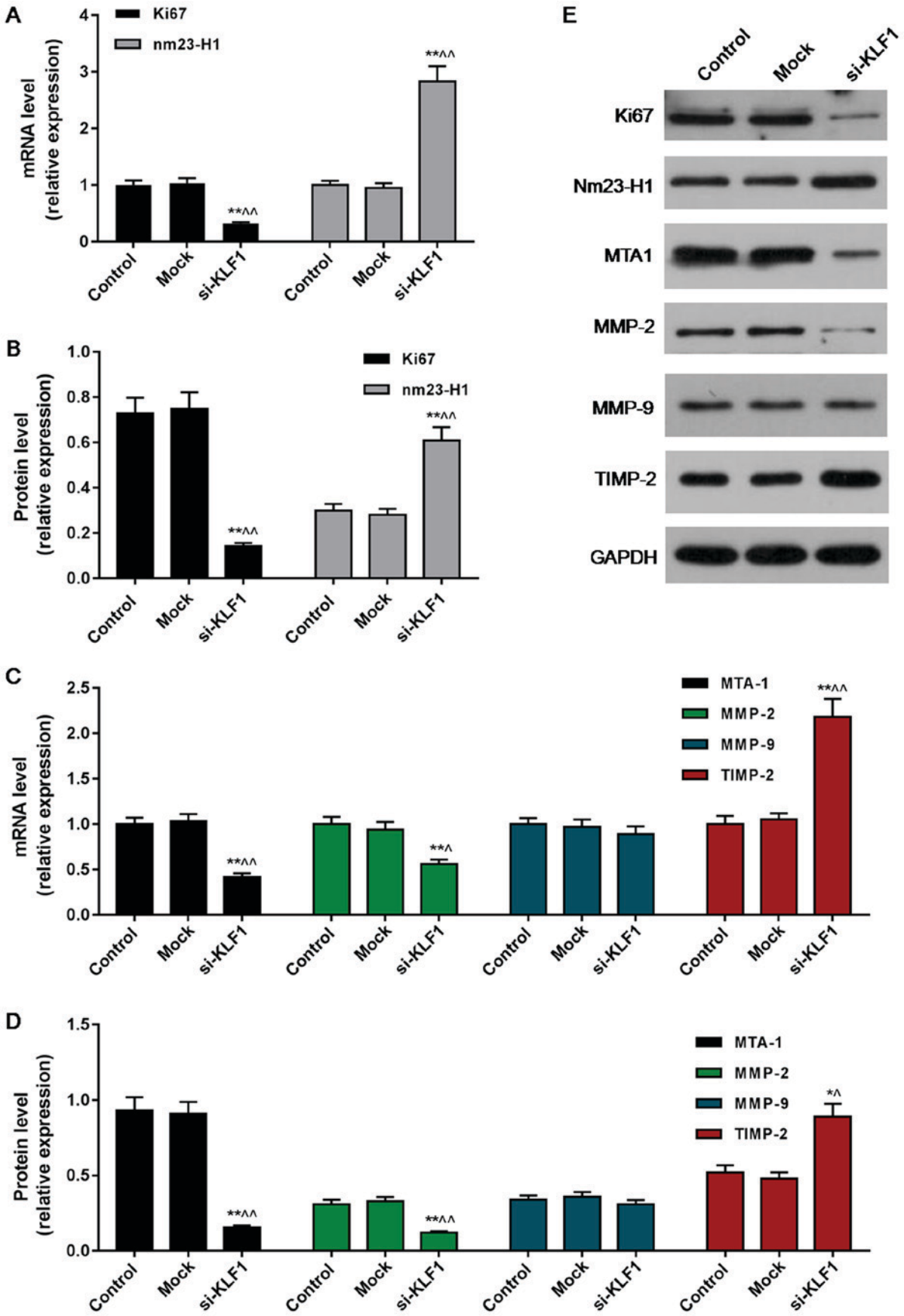


Figure 4. KLF1 interference influences the expression levels of cell proliferation and metastasis-associated factors. (A) The mRNA levels of Ki67 decreased, and nm23-H1 increased when KLF1 was silenced. (B and E) The protein levels of Ki67 decreased, and nm23-H1 increased when KLF1 was silenced. (C) The mRNA levels of MTA-1, MMP-2, MMP-9 and TIMP-2 were detected by reverse transcription-quantitative polymerase chain reaction. (D and E) The protein levels of MTA-1, MMP-2, MMP-9 and TIMP-2 were detected by western blotting. $^{*}$P<0.05 and $^{**}$P<0.01 vs. Control group; $^{\wedge}$P<0.05 and $^{\wedge\wedge}$P<0.01 vs. Mock group. KLF1, Krüppel-like factor 1; si-/siRNA, small interfering RNA; nm23-H1, nonmetastatic clone 23 type 1; MTA, metastasis-associated antigen; MMPs, matrix metalloproteinases; TIMP, tissue inhibitor of metalloproteinase-2.

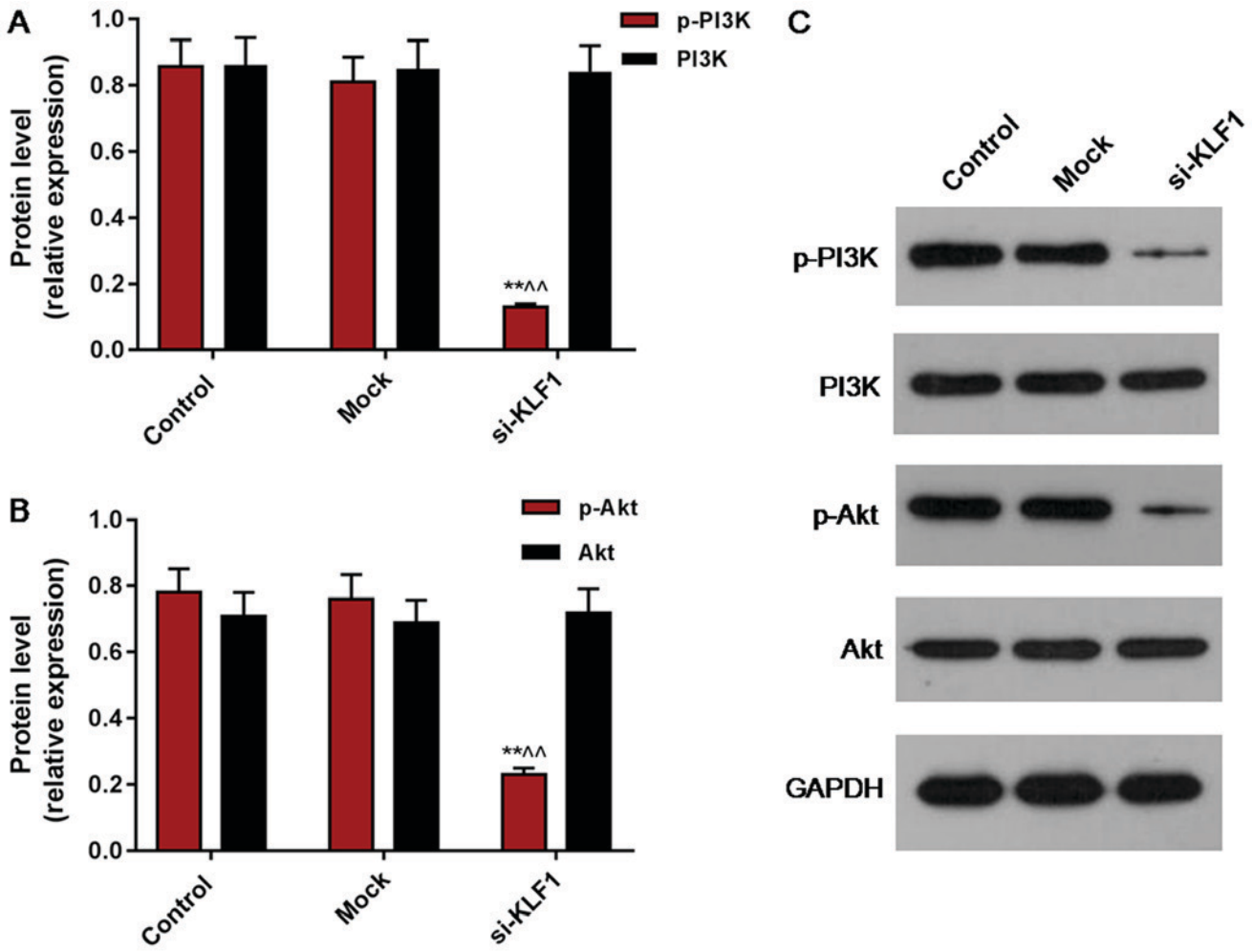


Figure 5. KLF1 interference inhibits the activation of the PI3K/Akt signaling pathway. The phosphorylation levels of (A and C) PI3K and (B and C) Akt proteins declined significantly in the KLF1-siRNA group. **P<0.01 vs. Control group; ^^P<0.01 vs. Mock group. KLF1, Krüppel-like factor 1; si-/siRNA, small interfering RNA; PI3K, phosphatidylinositol-3-kinase; Akt, protein kinase B; p-, phosphorylated.

inhibited by KLF1 knockdown. Therefore, we confirmed that KLF1 contributed to the inhibition of cervical cancer cell invasion and migration.

Being an important signaling pathway, PI3K signaling is involved in the regulation of cell proliferation, differentiation, apoptosis and it also has many other functions (36,37). Recent studies have found that the signaling pathway composed of PI3K and its downstream protein Akt was closely related to the development of tumors in human body (38,39). Mutations or abnormalities in some components of the signaling pathway can not only lead to the malignant transformation of the cell, but also to the migration of tumor cells, tumor angiogenesis and degradation of the extracellular matrix (40,41). Previous report has found that PI3K/Akt pathway was related to cell proliferation and the invasion of cervical cancer cells (42). The knockdown of KLF1 was found inhibiting the activation of PI3K/Akt pathway in our study, and such a phenomenon indicated that KLF1 promoted metastasis and invasion of cervical cancer via PI3K/Akt pathway.

In conclusion, KLF1 promotes metastasis and invasion via PI3K/Akt pathway in cervical cancer cells. However, the understanding of the function of the KLFs family is limited at present. Therefore, further researches on investigating the role of KLF1 in the pathogenesis of cervical cancer was required and the outcomes of such researches will provide new diagnosis markers and possible therapeutic targets for cervical cancer.

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**Availability of data and materials**

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

**Authors' contributions**

BiZ, QL and JC conceived and designed the study. QH, BoZ and QX acquired, analyzed and interpreted the data. BoZ and

JC drafted the manuscript and critically revised it for important intellectual content. All authors gave final approval of the version to be published. QH and QX agreed to be accountable for all aspects of the work in ensuring that questions related to the accuracy or integrity of the work are appropriately investigated and resolved. All authors read and approved the final manuscript.

**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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