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Research Article

Overexpression of PIP5KL1 suppresses the growth of human cervical cancer cells *in vitro* and *in vivo*

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Abstract

PIP5KL1 (phosphatidylinositol-4-phosphate 5-kinase-like 1), the fourth member of PIP5Ks (phosphatidylinositol-4-phosphate 5-kinases) type I, acts as a scaffold for localization and activation of PIP5Ks, which in turn regulate numerous cellular processes. However, the role of PIP5KL1 in the development of human cancer is poorly studied. In this study, we established a stable clone of PIP5KL1 overexpressing human cervical cancer HeLa cells. RT-PCR (reverse transcription-polymerase chain reaction) and Western immunoblot analysis were performed to testify the mRNA and protein levels of PIP5KL1 in HeLa cells. The effect of PIP5KL1 overexpression on *in vitro* cell growth was assessed by measuring cell proliferation and migration. The athymic nude mouse model was used to examine the effects of PIP5KL1 on tumour growth *in vivo*. Stable transfection of PIP5KL1 induced a significant increase in expression of both mRNA and protein levels and consequent robust inhibition of proliferation ($P<0.05$) and migration ($P<0.05$) of HeLa cells. Overexpression of PIP5KL1 significantly suppressed the growth of HeLa xenograft tumours in the flanks of nude mice. Taken together, these studies indicate a functional negative correlation between elevated levels of PIP5KL1 and the development of human cervical cancer, suggesting that PIP5KL1 overexpression may suppress cervical cancer formation.

Keywords: human cervical cancer; migration; nude mice; PIP5KL1; proliferation

1. Introduction

Cervical cancer is one of the most common types of cancer in women worldwide, and knowledge regarding its cause and pathogenesis is expanding rapidly. There is conclusive evidence of the linkage between high-risk HPV (human papillomaviruses) and cervical cancer (Schiffman et al., 2007; Oaknin and Barretina, 2008). But, HPV infection is not sufficient for the development of cervical cancer because only a small fraction of those infected develop cancer (Alvarez-Salas and DiPaolo, 2007), suggesting the contribution of other factors to the progression of cervical cancer.

PIPKs (phosphatidylinositol phosphate kinases) act as a unique family of enzymes that generate the important secondary messenger PI(4,5)P (phosphatidylinositol 4,5-bisphosphate), which plays critical roles in the regulation of numerous cellular processes. Two types of phosphoinositide kinase, designated type I PIPKs [also named as PIP5K (phosphatidylinositol 4-phosphate 5-kinases)], and type II PIPKs [also named as PIP4K (phosphatidylinositol 5-phosphate 4-kinases)] have been well characterized. Both type I and II PIPKs consist of α, β and γ isoforms (Pendaries et al., 2003; Stace et al., 2008). Because of their close correlation with the development of human cancers, these enzymes and their function have gained much attention.

Accumulating evidence indicates that PIPKs participate in the regulation of cancer cell migration, adhesion and invasion (Luoh et al., 2004; Sun et al., 2007). It is demonstrated that in different subsets of breast cancer cell lines, overexpression of PIP5K2β increases breast cancer cell proliferation and anchorage-independent growth (Luoh et al., 2004). Similarly, another study indicates that PIP5Kγ is required for growth factor-stimulated cell migration in HeLa and A431 cells (Sun et al., 2007). These findings greatly suggest a possible functional linkage between PIPKs and cancer development.

Recently, PIP5KL1 [(phosphatidylinositol 4-phosphate 5-kinase-like 1) also named as PIPKH (phosphatidylinositol phosphate kinase homologue)], was identified as the fourth member of PIP5K by searching the human genome sequence and EST databases (Wang et al., 2006). Our previous TMA (tissue microarray) data indicated that this new member was rarely expressed in the cervical (L. Shi, M. Zhao and C. Huang, unpublished data) and gastric cancer tissues. Furthermore, overexpression of PIP5KL1 significantly suppressed human gastric cancer BCG823 cell

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Abbreviations: DAPI, 4,6-diamidino-2-phenylindole; FBS, fetal bovine serum; GFP, green fluorescent protein; HPV, high-risk human papillomaviruses; MTT, 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide; PIPK, phosphatidylinositol phosphate kinase; PIPKH, phosphatidylinositol phosphate kinase homolog; PIP4K, phosphatidylinositol 5-phosphate 4-kinases; PIP5K, phosphatidylinositol 4-phosphate 5-kinases; PIP5KL1, phosphatidylinositol 4-phosphate 5-kinase-like 1; PIP5Ks, phosphatidylinositol-4-phosphate 5-kinases; PI(3,4,5)P, phosphatidylinositol-3,4,5-bisphosphate; PI(4,5)P, phosphatidylinositol 4,5-bisphosphate; RPMI, Roswell Park Memorial Institute; RT-PCR, reverse transcription-polymerase chain reaction; TMA, tissue microarray.

proliferation and migration, indicating a functional negative correlation between PIP5KL1 overexpression and human gastric cancer development (Shi et al., 2009). But the precise role of PIP5KL1 in cervical cancer development is still lacking.

In this study, we established a stable clone of PIP5KL1-overexpressing human cervical cancer HeLa cells and presented the direct evidence that overexpression of PIP5KL1 at both transcriptional and translational levels inhibited the proliferation and migration of HeLa cells. Moreover, PIP5KL1 overexpression significantly suppressed the growth of HeLa xenograft tumours in nude mice. This suggested that PIP5KL1 appears to be a potential tumour suppressor in cervical cancer development. To the best of our knowledge, this is the first report revealing the role of PIP5KL1 in human cervical cancer development.

2. Materials and methods

2.1. Cell culture

HeLa, a human cervical carcinoma cell line, was maintained in RPMI (Roswell Park Memorial Institute) 1640 (Gibco) supplemented with 10% heat-inactivated FBS (fetal bovine serum) (Hyclone), 100 units/ml penicillin and 100 μg/ml streptomycin. Cells were grown at 37℃ in a humidified 5% CO_2 atmosphere and used for assays during the exponential phase of growth.

2.2. Plasmid transfection and generation of stable PIP5KL1 transfectants

Human PIP5KL1 cDNA (GenBank # NM_173492) was subcloned into the pcDNA3.1 and pEGFPC1 eukaryotic expression vectors. Transfection of the recombinant vector was performed by Lipofectamine (Invitrogen) according to the manufacturer's protocol. At 24 h after transfection, the selection medium with 600 μg/ml G418 (Gibco) was added and replaced every other day, to remove dead cells, for 20–25 days until G418-resistant colonies were of sufficient size to be plated in 24-well culture plates. After 2 months of screening and culture, several colonies expressing PIP5KL1 were established.

2.3. Immunofluorescence imaging analysis

Cells seeded in chamber slides were transfected with plasmid DNA for 24 h. After fixation with freshly prepared 4% paraformaldehyde in PBS for 15 min, cells were subsequently permeabilized with 0.1% Triton X-100 and blocked with PBS containing 2% BSA for 1 h. For immunostaining, cells were incubated with anti-PIP5KL1 antibody for 1 h at room temperature (24℃) and then incubated with TRITC-conjugated anti-rabbit IgG (Santa Cruz Biotechnology) for 30 min. The nuclei of cells were stained with DAPI (4,6-diamidino-2-phenylindole) and then examined under a TCS-SP2 confocal microscope (Leica Microsystems).

2.4. RNA extraction and RT-PCR (reverse transcription-polymerase chain reaction)

Total RNA was prepared using Trizol reagent (Life Technologies, Inc.) according to the manufacturer's instructions. Four micrograms of total RNA were used to synthesize the first strand of cDNA using cDNA Synthesis Kit (Fermentas). The primer sequences of PIP5KL1 were used as follows: sense 5′-AGGCTATCAGGAGGCTGTAATC-3′ and antisense 5′-TTCAGGGCAAGACCATCAAC-3′. β-Actin was used to ascertain the equal amount of cDNA in each reaction. A total of 30 cycles were performed with each cycle consisting of 30 s at 94℃, 35 s at 65℃ and 40 s at 72℃ with an initial denaturation of 5 min at 95℃ and a final extension of 7 min at 72℃. The reaction products were separated on 1% agarose gel and analysed by an imaging system.

2.5. Western immunoblot analysis

Cells were pelleted by centrifugation, lysed in lysis buffer (10 mM Hepes, pH 7.9, 1.5 mM MgCl, 10 mM KCl, 1 mM EDTA, 1 mM EGTA, 1% NP-40, 5mg/ml leupeptin, 5 mg/ml aprotinin, 1 mM PMSF) for 30 min on ice. Lysates were clarified by centrifugation at 18,000 ***g*** for 10 min at 4℃. The protein concentrations in the supernatants of the lysates were determined using the BCA (bicinchoninic acid) protein assay reagent (Thermo Fisher Scientific). Equal amounts of protein (20 μg) were separated on SDS/PAGE (12% gels) and electroblotted on to PVDF membranes (Pall Corporation). Membranes were blocked in Tris-buffered saline containing 0.05% TBS-T (Tween-20) and 5% non-fat milk for 1 h and incubated with the anti-PIP5KL1 antibody (established in our laboratory) overnight at 4℃. Blots were washed three times for 10 min each with TBS-T and incubated for 1 h in the dark with the appropriate IRDyeTM 800-conjugated secondary antibodies (Santa Cruz Biotechnology) prepared in TBST/5% non-fat milk. All bands were detected using ECL Western blot kit (Amersham Life Science Inc.) and quantified by scanning densitometry (model GS 670; Bio-Rad).

2.6. MTT assay

The MTT [3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide] assay was performed as previously described with the following modifications (Do et al., 2008). Briefly, cells were seeded into 96-well plates with 0.6×10^4 cells/well and maintained in 200 μl of RPMI 1640 medium containing 10% FBS. At the indicated time points, cells were washed with PBS, and 10 μl of MTT (5 mg/ml) was added to each well and further incubated for 4 h. The MTT solution was carefully removed by aspirating, and the formazan product was dissolved in 150 ml DMSO. Absorbance was measured at 570 nm on a Shimadzu Graphicord UV-240 spectrophotometer (Shimadzu). All MTT assays were repeated four times.

2.7. Wound closure assay

Cells were cultured in a six-well culture plate in RPMI 1640 supplemented with 10% FBS. After reaching confluence, a scratch wound was carried out by creating a linear cell-free region using a p200 pipette tip. The debris was removed and the

edge of the scratch was smoothed by washing the cells twice with PBS and the cells were re-fed with RPMI 1640 medium containing 1% BSA. Then the dishes were placed in the incubator for 48 h. The progress of cell migration into the scratch was photographed at 0 and 48 h using an inverted microscope. The images were further analysed quantitatively by using National Institutes of Health ImageJ software.

2.8. Boyden chamber assay

Boyden chamber assay was performed using 24-well transwell chamber with 8.0 μm pore polycarbonate filter inserts (Costar). Briefly, cells (1×10^4 cells/well) suspended in serum-free RPMI 1640 containing 0.1% BSA were overlaid in the upper chamber of each transwell. In each lower chamber, 600 μl of RPMI 1640 supplemented with 10% FBS was added. Then the inserts were incubated at 37°C in a humidified atmosphere containing 5% CO_2 for 24 h. The cells that had not penetrated the filters were removed using cotton swabs. The migrated cells attached to the bottom side were fixed in 100% methanol for 10 min and stained in 0.2% crystal violet for 20 min, rinsed in PBS and examined under a bright-field microscope with $\times 10$ magnification. The value of migratory activity was expressed as the average number of migrated cells per microscopic field over the five fields in each assay from four independent experiments.

2.9. Tumour growth in nude mice

HeLa cells transfected with either pCDNA3.1 or pCDNA3.1-PIP5KL1 were selected in 600 μg/ml of G418. Twelve nude mice (male, 4–5 weeks old, from Cancer Institute and Hospital, Chinese Academy of Medical Sciences, Beijing, China) were randomly divided into six animals in the treated group and six animals in the control group. Mouse experiments were approved by the Institutional Review Board of Animal Care of the Animal Center, Peking Union Medical College and Chinese Academy of Medical Sciences. Approximately one million cells (pcDNA3.1 transfected HeLa cells for control group or pcDNA3.1-PIP5KL1 transfected HeLa cells for treated group) were injected into the flank of each mouse to initiate tumour growth. Seven days after inoculation, tumour size was measured every week by using a dial caliper, and tumour volumes were calculated as [length $\times$ (width^2)]/2. After 28 days, the experiment was terminated, and the tumours were then excised. The weights of tumours were measured. The tissues were frozen in liquid nitrogen and stored at −80°C for RT-PCR detection.

2.10. Statistical analysis

Statistical analyses were performed using the statistical software SPSS 12.0 (SPSS Inc.). Data were expressed as means ± S.E.M. Student's *t* test was used for statistical comparison. Cell proliferation and the migration assay were tested using one-way ANOVA (analysis of variance). Statistical significance was defined as $P<0.05$ in all analyses.

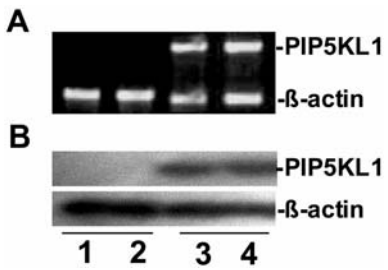

Figure 1 Expression of PIP5KL1 in HeLa cells by RT-PCR and Western immunoblot analysis

Cells were transfected with pcDNA3.1 (lanes 1 and 2) or pcDNA3.1-PIP5KL1 (lanes 3 and 4). After 24 h of transfection and antibiotic selection, total RNA and protein were prepared for expression analyses by (**A**) RT-PCR and (**B**) Western immunoblot.

3. Results

3.1. Expression of PIP5KL1 in HeLa cells

To investigate the biological effect induced by PIP5KL1, HeLa cells, which have very low PIP5KL1 levels, were stably transfected with a full-length PIP5KL1 cDNA construct (pcDNA3.1-PIP5KL1). Two PIP5KL1-expressing clones were determined by RT-PCR and Western blot analysis after antibiotic selection. Compared with pcDNA3.1 transfected cells, the levels of PIP5KL1 mRNA and protein were greatly increased in PIP5KL1 transfectants (Figures 1A and 1B).

3.2. The subcellular localization of PIP5KL1

To determine the subcellular localization of PIP5KL1, we expressed GFP-PIP5KL1 fusion proteins in HeLa cells. Cells transfected with pEGFPC1-PIP5KL1 showed a clear GFP (green fluorescent protein) signal in the perinuclear area (Figure 2b), whereas cells transfected with pEGFPC1 displayed a diffuse GFP signal throughout the cell (Figure 2a). In addition, to exclude the effect of GFP on the PIP5KL1 localization, we used another recombinant expression vector pcDNA3.1-PIP5KL1. When transfected HeLa cells were incubated with anti-PIP5KL1 antibody followed by a TRITC-conjugated secondary antibody, the fluorescence intensity which represented the PIP5KL1 expression level displayed a significant promotion in the perinuclear area (Figure 2d). This suggested that PIP5KL1 proteins are mostly localized to the perinuclear area.

3.3. Overexpression of PIP5KL1 inhibits the proliferation of HeLa cells *in vitro*

To examine the effect of PIP5KL1 on HeLa cell proliferation, cells were stably transfected with pcDNA3.1 and pcDNA3.1-PIP5KL1, respectively. The difference in cell growth inhibition rate between PIP5KL1-transfected cells from other groups was not statistically significant in the first 2 days. The significant growth inhibition in PIP5KL1-transfected HeLa cells was detected from the third day and that was markedly lower ($P<0.01$) than that in pcDNA3.1-transfected HeLa cells and that in control HeLa cells (Figure 3).

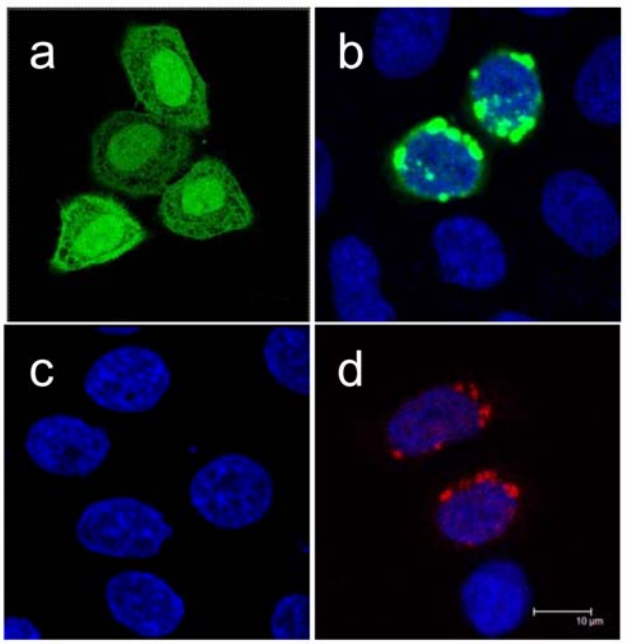

Figure 2 Expression and subcellular distribution of PIP5KL1 in HeLa cells identified by immunofluorescence staining

Cells were transiently transfected with (**a**) pEGFPC1, (**b**) pEGFPC1-PIP5KL1, (**c**) pcDNA3.1 empty plasmid and (**d**) pcDNA3.1-PIP5KL1 recombinant plasmids. GFP and GFP-PIP5KL1 expression was directly observed by fluorescence microscopy, and PIP5KL1 expression was detected by immunofluorescence staining using anti-PIP5KL1 antibody.

3.4. Overexpression of PIP5KL1 inihibits the migration of HeLa cells *in vitro*

A wound healing assay was performed to assess whether PIP5KL1 overexpression is associated with the HeLa cell migration. We found that PIP5KL1-overexpressing cells

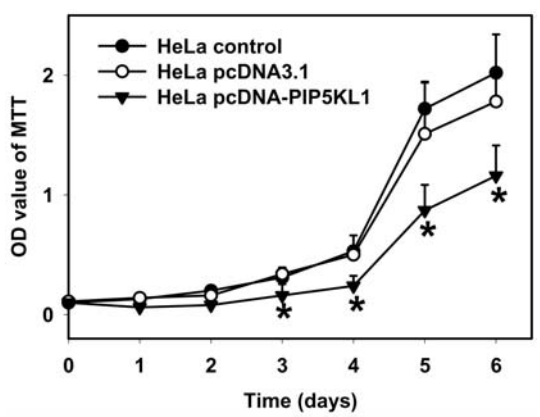

Figure 3 Effects of PIP5KL1 overexpression on HeLa cell proliferation by MTT assay

Cells were seeded into 96-well plates and maintained in RPMI 1640 containing 10% FBS. At the indicated time points, cell number was determined with the MTT assay, and the graph shows the results of four independent experiments. *Results differ ($P<0.05$) from the HeLa control and HeLa pcDNA3.1.

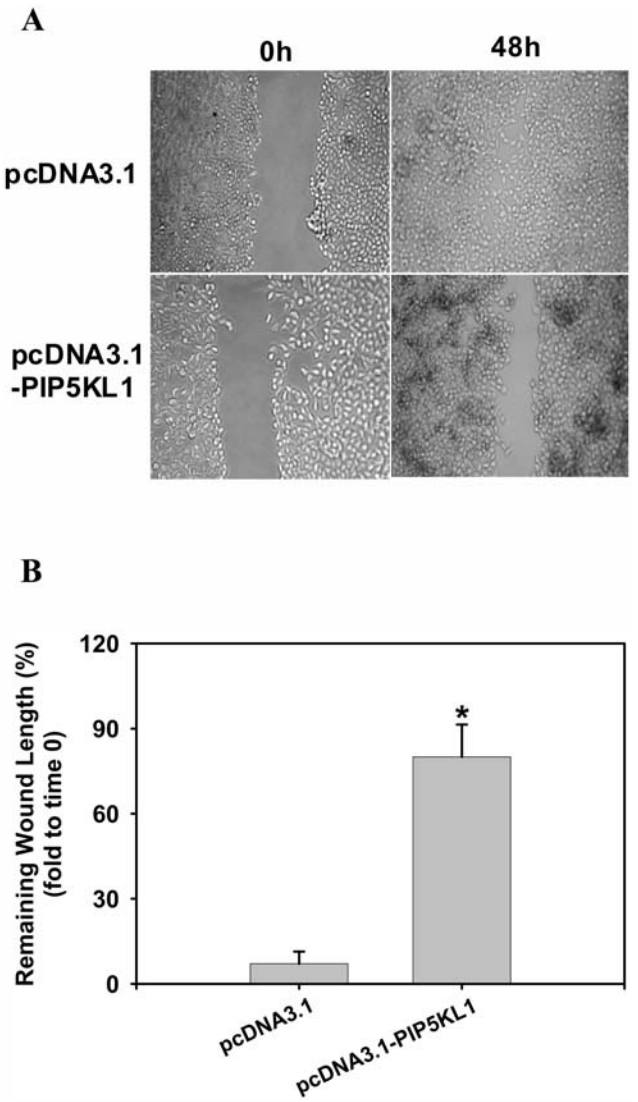

Figure 4 Effects of PIP5KL1 overexpression on HeLa cell migration by wound healing assay

Confluent monolayer cells were subjected to scratch wounding as described in the Materials and methods section. Then cells were incubated in RPMI 1640 serum-free medium supplemented with 1% BSA. (**A**) After 48 h migration, wound closure was photographed and further analysed quantitatively. (**B**) Quantified data are expressed as means ± S.E.M. from three independent experiments. *Results differ ($P<0.05$) from the HeLa pcDNA3.1 cells.

(pcDNA3.1-PIP5KL1) displayed a significant decrease in cell migration ability when compared with the pcDNA3.1-transfected cells (Figure 4). It is noteworthy that this wound healing assay is a combination of migration and proliferation based on a review by Yarrow et al. (2004). To exclude the possibility that PIP5KL1-inhibited cell proliferation plays a significant role in this migration assay, we further performed the chamber transwell assay. Consistent with the above results, the migratory ability of the PIP5KL1-overexpressing cells was significantly suppressed in comparison with the pcDNA3.1 vector-transfected HeLa cells (Figure 5). This indicates that overexpression of PIP5KL1 is negatively correlated with the migration of HeLa cells.

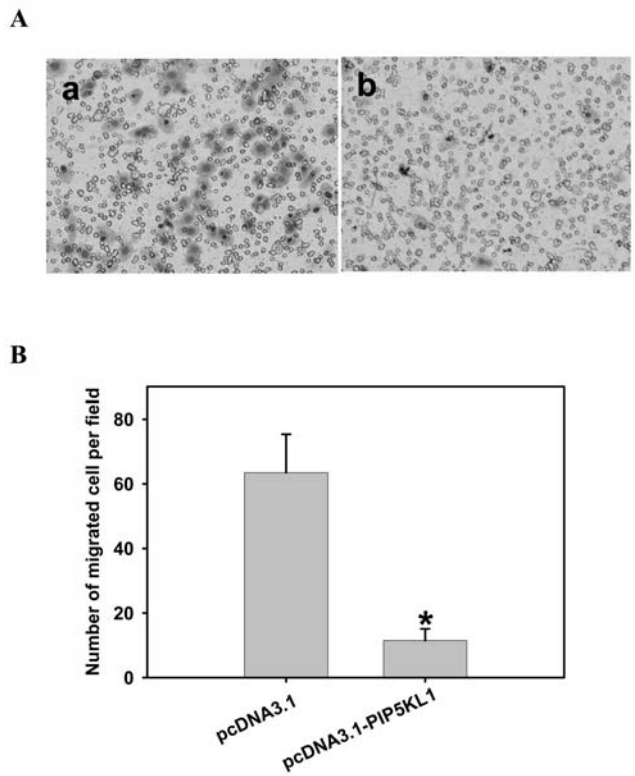

Figure 5 Effects of PIP5KL1 overexpression on HeLa cell migration by transwell chamber assay

Cells were overlaid in the upper chambers of 24-well tissue culture plates containing RPMI 1640 supplemented with 10% FBS. After incubation for 24 h, penetrating cells were fixed and stained with 0.1% crystal violet. (**A**) Photographs depict migration of HeLa pcDNA3.1 cells (a) and HeLa pcDNA3.1-PIP5KL1 cells (b). (**B**) Quantified data are expressed as means $\pm$ S.E.M. from four independent experiments. *Results differ ($P<0.05$) from the HeLa pcDNA3.1 cells.

3.5. Overexpression of PIP5KL1 inhibits tumour growth in nude mice

The *in vitro* assays suggest that tumorigenicity *in vivo* may be inhibited by PIP5KL1 overexpression. To examine this possibility, we performed *in vivo* tumorigenesis assays in the nude mice. HeLa cells stably transfected with either pcDNA3.1-PIP5KL1 or pcDNA3.1 vector were injected into nude mice. As shown in Figures 6(A) and 6(B), the growth rate of tumours in mice injected with PIP5KL1-pcDNA-transfected HeLa cells was significantly suppressed from day 14 through to day 28 after inoculation when compared with pcDNA3.1-transfected cell-injected mice. The weight of tumour at day 28 after inoculation showed significant decrease in the mice injected with PIP5KL1-pcDNA-transfected HeLa cells (Figure 6C). As expected, RT-PCR analysis confirmed high PIP5KL1 mRNA expression in the tumours injected with pcDNA3.1-PIP5KL1 transfected cells and no PIP5KL1 expression in the tumours injected with control cells (Figure 6D). Together, these results showed that overexpression of PIP5KL1 suppresses the growth of xenograft tumour *in vivo*.

4. Discussion

In our previous studies, we observed that PIP5KL1 expression was negatively correlated with human gastric cancer development. To further elucidate the effect of PIP5KL1 on cervical cancer progression, we transfected PIP5KL1 cDNA into a highly malignant cervical cancer HeLa cell line, which has very low levels of endogenous PIP5KL1 expression. Our data indicated that PIP5KL1 overexpression at both transcriptional and translational levels inhibited cell proliferation ($P<0.05$) and migration ($P<0.05$) in HeLa cells. Furthermore, PIP5KL1 overexpression significantly suppressed the growth of xenograft tumours in nude mice models. This suggested that PIP5KL1 has a potential tumour-suppressive function in cervical tumour formation. To our knowledge, this is the first report of a functional negative correlation between elevated levels of PIP5KL1 and the development of human cervical cancer.

It is well known that cell proliferation and migration play essential roles in the tumour formation process. PIPKs can utilize PI(4)P or PI(5)P as substrates to generate the majority of PI(4,5)P, which in turn participates in the regulation of cell motility/cytoskeletal assembly and cell migration (Honda et al., 1999; Heck et al., 2007). Recent evidence suggests that specific isoforms of type I PIPKs are involved in different aspects of migration, including cortical actin organization (Coppolino et al., 2002), membrane ruffle formation (Yamamoto et al., 2001) and the assembly/disassembly of focal adhesions (Ling et al., 2002). Furthermore, these different isoforms and their specific subcellular distribution can make them have distinct roles in cell migration progress. Although PIP5KL1 has only a very low intrinsic phosphoinositide kinase activity, it still markedly increases PI(4,5)P2 levels as a scaffold to localize and activate other PIPKs isoforms (Chang et al., 2004). From our present study, PIP5KL1 appeared to play a negative role in the regulation of HeLa cell migration. This is in contrast with the effect of PIPKIα on MEF (mouse embryonic fibroblast) migration. It was found that PIPKIα had a positive effect on MEF motility, and depletion of PIPKIα in MEFs blocked its migration (Kisseleva et al., 2005). Similarly, another report also indicated that PIP5Kγ performed positively on growth factor-stimulated HeLa and A431 cell migration. A probable explanation for this difference is owing to their specific subcellular distribution, which directly determines the kinase activity of PIPKs (Ling et al., 2006). It is known that PIPKIα is mainly present in the cytoplasm and nucleus (Coppolino et al., 2002), and PIPKIγ localizes to the cytoplasm membrane (Di Paolo et al., 2002; Ling et al., 2002), while PIP5KL1 is mostly localized to the perinuclear region in HeLa cells from our present data. In addition, different signalling pathways that participate in the regulation of PIPK activity may also be involved in this distinct action.

Unlike cell migration, the role of PIPKs in cell proliferation still remains unclear. It is reported that PIP5KL1 was closely associated with cell viability by using cell-based high-throughput assay, and PIP5KL1 induced 293 T-cell necrosis and apoptosis (Wang et al., 2006). In this study, we also observed that PIP5KL1 expression robustly inhibited HeLa cell proliferation. But whether

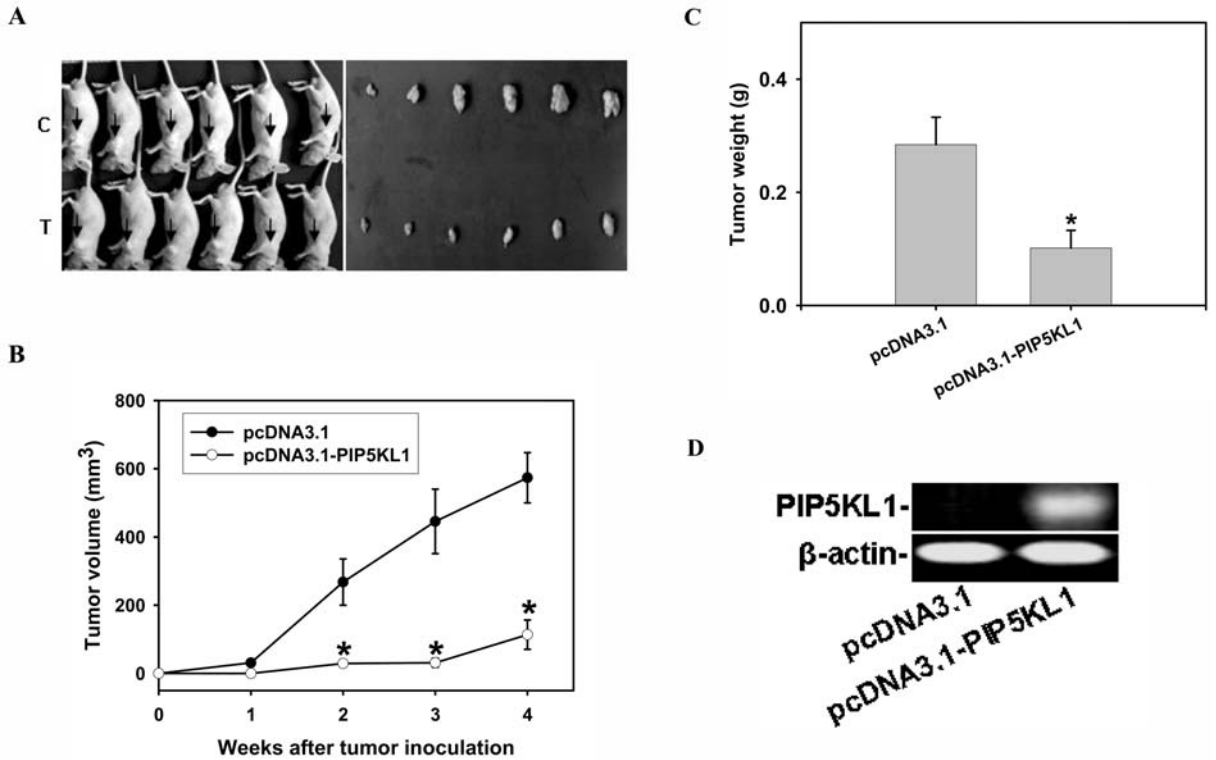

Figure 6 **Effect of PIP5KL1 overexpression on tumour growth in nude mice**

Twelve animals were randomly divided into six animals in the treated group and six animals in the control group. Approximately one million cells (pcDNA3.1-transfected HeLa cells for control group or pcDNA3.1-PIP5KL1-transfected HeLa cells for treated group) were injected into the flank of each mouse to initiate tumour growth. Once tumours started to grow, their sizes were measured weekly and the tumour volume was calculated. (**A**) Left, photographs of representative mice with tumours from each group; right, photographs of excised tumours from each group. C, control group; T, treated group. (**B**) Average tumour volume of control and treated group after tumour cell inoculation. (**C**) Average tumour weight of control and treated group after 28-days inoculation. (**D**) Expression of PIP5KL1 mRNA in xenografted tumours as determined by RT-PCR. Results are expressed as tumour volume (mm^3) $\pm$ S.E.M. of six mice. *$P<0.05$ was considered to be statistically significant compared with the control group.

this suppression effect is associated with cell necrosis and apoptosis still requires us to further testify.

In the light of the above data obtained from an *in vitro* experiment, we hypothesized that PIP5KL1 might exert suppression on *in vivo* tumour growth. As expected, tumorigenicity assay in nude mice showed significant suppression in tumour growth, which probably contributed to the PIP5KL1-induced suppression effect on cell proliferation and migration.

In conclusion, this study, to our knowledge, is the first systemic one to investigate the effects of PIP5KL1 on the development of cervical cancers *in vitro* and *in vivo*. These findings may implicate, at least partially, that PIP5KL1 could represent an appropriate target for drug treatment of tumours. Subsequent analysis is needed to further clarify the function and regulation of PIP5KL1 in cancer development and progression.

Author contribution

Lan Shi was in charge of the concept and design, collection and assembly of data, data analysis and interpretation, and manuscript writing. Kai Wang performed the data analysis and interpretation, and manuscript writing. Mei Zhao was in charge of the provision of study material and animals, collection and assembly of data. Xinghua Yuan performed the collection and assembly of data. Changzhi Huang was in charge of the concept and design, data analysis and interpretation, and final approval of manuscript.

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