

TAT-mediated si-hWAPL inhibits the invasion and metastasis of cervical cancer stem cells

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Abstract. Human wings apart-like (hWAPL) is reported to have an association with cervical cancer. In the present study, the role of hWAPL in cervical cancer stem cells (CCSCs) was evaluated. Cervical tumorspheres were generated from cervical cancer tissues cultured in stem cell medium, and the expression of hWAPL by the tumorspheres was detected using immunohistochemistry. hWAPL expression levels in the tumorspheres were then upregulated using hWAPL adenoviral vectors or downregulated via the TAT-mediated knockdown of hWAPL and the effects on the tumorspheres were evaluated using colony formation, cell invasion and western blotting assays. The results demonstrated that the expression of hWAPL and human papillomavirus (HPV) was associated with the pluripotency of CCSCs, with hWAPL expression decreasing following the differentiation of cervical tumorspheres. Knockdown of hWAPL expression decreased HPV E6 expression and inhibited tumor invasion and colony formation. TAT-mediated knockdown of hWAPL with short interfering RNA significantly reduced tumor growth in nude mice. These results suggest that hWAPL is a marker of CCSC proliferation and is potentially a therapeutic target for cervical carcinoma through the downregulation of HPV E6.

Introduction

Cervical cancer is one of most common cancers in females and the second most common cause of cancer-related mortality in women in China; its mortality varies among provinces, with the highest mortality rates ranging from 7.28 to 11.88/10,000 females

in poorly developed areas (1). The poor prognosis of cervical cancer (including renal failure and metastasis) is associated with its highly invasive and diffusely metastatic characteristics (2,3). Human papillomavirus (HPV) has been recognized to cause cervical carcinogenesis (1). The mechanism by which HPV induces tumor formation is considered to involve the binding of the viral oncoproteins HPV E6 and E7 to the tumor suppressors p53 and retinoblastoma protein, respectively (4).

Cervical cancer stem cells (CCSCs) are thought to be the 'seed cells' in cancer metastasis and recurrence (5-9). A number of studies have shown that CCSCs and core stem cell transcription factors (TFs) such as forkhead box D3 (5), sex-determining region Y-box 9 protein (Sox9) (6), Sox2 (7,8), Nanog (9) and octamer-binding protein 4 (Oct4) (8) are highly expressed in cervical cancer. These TFs are recognized as stem cell markers because they maintain the pluripotency of stem cells (10,11). Recently, Tyagi *et al* (12) reported that HPV E6 is overexpressed in CCSCs, indicating that it plays a role in maintaining the pluripotency of these cells.

The human wings apart-like (hWAPL) gene is a homologous sequence of the *Drosophila* WAPL gene, and is closely associated with cervical carcinogenesis (13). HPV E6 and E7 are able to induce high levels of hWAPL expression, which plays a key role during the development of cervical cancer (14). Therefore, in the present study, the expression of hWAPL in CCSCs was evaluated, and its effects on invasion and colony formation in this cell population were investigated.

Materials and methods

Culture of tumorspheres derived from CCSCs. The study protocol was approved by the Medical Ethics Committees of Xi'an Jiaotong University (no. H34-32-1; Xi'an, China). All experiments were conducted in accordance with the Declaration of Helsinki. The method of culturing tumorspheres derived from CCSCs was conducted as previously reported (15). Briefly, 17 samples of cervical cancer tissue (stage IB, n=11; stage IC, n=3; stage IIa, n=3; patient age, 43-65 years) were obtained by resection. These samples were positive for HPV E6 expression, as determined by western blot analysis. To prepare the cervical tumorspheres (CTs), tumor tissues were washed immediately with PBS and digested overnight in Dulbecco's modified Eagle's medium (DMEM)/F12; v/v, 1:1; Gibco; Thermo Fisher Scientific,

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Inc., Waltham, MA, USA) supplemented with 0.5 mg/ml collagenase IV (Gibco; Thermo Fisher Scientific, Inc.). The digested tissues were then cultured in stem cell medium [DMEM/F12 with 10 ng/ml basic fibroblast growth factor (bFGF), 10 U/ml leukemia inhibitory factor, 1×10^5 U/l penicillin and 100 mg/l streptomycin; all from Merck KGaA, Darmstadt, Germany] at 37°C in a humidified atmosphere containing 5% CO₂. Clones of >50 cells were recognized as tumorspheres, and were dissociated every 7-10 days by incubation in a non-enzymatic cell dissociation solution (Sigma-Aldrich; Merck KGaA) for 2 min at 37°C, and passaged at a density of 1×10^3 cells/100-mm plate. CTs were completely differentiated by 8 days after switching to stem cell medium without bFGF.

Transduction with adenoviral vectors. All adenoviral vectors (Ad5 serotype, E1/E3 deficiency double DNA; constructed by Beijing Nuosai Genome Research Center Co., Ltd., Beijing, China) used had comparable titers of 10^8 - 10^9 transducing U/ml. Suspensions of the vectors were stored at -80°C until use. The primers used for hWAPL overexpression are shown in Table I. Suspensions were briefly centrifuged (500 x g, room temperature) and kept on ice immediately prior to use. For transduction, 2×10^4 dissociated tumorspheres were transduced 1 day after the initial seeding of cells with a multiplicity of infection of 25. Cells were incubated in stem cell medium containing adenoviral particles and 4 µg/ml Polybrene (Santa Cruz Biotechnology, Inc., Dallas, TX, USA) for 18 h at 37°C in a humidified atmosphere containing 5% CO₂. Adenoviral particles were removed, and the medium was replaced with fresh stem cell medium.

Colony formation assay. A colony formation assay was performed as previously described (15). Briefly, single cervical carcinoma cells or dissociated tumorspheres were cultured in DMEM/F-12 medium supplemented with 10% fetal calf serum (FCS; Thermo Fisher Scientific, Inc.), 2 mmol/l glutamine (Invitrogen; Thermo Fisher Scientific, Inc.), 1×10^5 U/l penicillin and 100 mg/l streptomycin. Cells were cultured at clonal densities of 100-300/cm² on 2% gelatin (Sigma-Aldrich; Merck KGaA)-coated tissue culture dishes (BD Biosciences, San Jose, CA, USA) at 37°C in 5% CO₂ in air. Clones were monitored every day, and the medium was changed every 2-3 days. After 28 days of culture, plates were fixed in 10% formaldehyde/PBS for 10 min and stained with Harris hematoxylin. Clones (>50 cells) were counted for ≥3 plates per sample and averaged. The efficiency of colony formation was determined as follows: Efficiency (%) = number of colonies/number of cells seeded x 100.

Implantation of tumorsphere-derived cells into nude mice. Following the dissociation of tumorspheres from 17 cervical cancer patients in a non-enzymatic cell-dissociation solution, cells were washed in serum-free Hank's balanced salt solution. Cells were then suspended in a 1:1 (v/v) mixture of serum-free DMEM/F12, and 1×10^5 cells were injected subcutaneously into the right and left sides of the mid-abdominal area of nude mice using a 23-G needle. Animals were subjected to dissection and analysis 28 days after implantation, and tumor growth was assessed by measurement of the tumor volume (V) using the formula: $V = 1/2 \times (L \times W^2)$, where L is the length of the tumor and W is the width.

Western blotting. To extract the total protein, 1×10^4 cells were lysed in lysis buffer [1.0 M Tris-HCl (pH 6.8) 1.0 ml, 10% SDS 6.0 ml, β-mercaptoethanol 0.2 ml and ddH₂O 2.8 ml] on ice for 10 min. The lysate was then subjected to centrifugation at 10,000 x g at 4°C for 10 min. Following protein denaturation at 100°C for 10 min, the protein level was normalized by measuring the absorbance at 280 nm. Then, 80 µg protein samples were analyzed by 12.5% SDS-PAGE and gels were transferred onto an Immobilon-P transfer membrane (polyvinylidene difluoride; EMD Millipore, Billerica, MA, USA). The membrane was blocked with 5% non-fat dried milk in TBST (10 mM Tris-HCl, 150 mM NaCl and 0.1% Tween-20) for 1 h at room temperature, and incubated overnight at 4°C with anti-HPV E6, Oct4 and hWAPL antibodies (1:100 dilution in PBS, cat. nos. sc-460, sc-101534 and sc-365189, respectively) or GAPDH (1:500 dilution, cat. no. sc-293335) (both from Santa Cruz Biotechnology, Inc.). Following incubation with horseradish peroxidase (HRP)-labeled rabbit anti-mouse secondary antibodies (1:2,000 dilution, cat. no. sc-358919; Santa Cruz Biotechnology, Inc.), membranes were developed using a SuperSignal® West Pico Trial kit (Pierce; Thermo Fisher Scientific, Inc.). Protein quantitation was conducted from the optical density using software for Bio-Rad's Molecular Imager® systems (Image Lab™ 2.0 and Molecular Imager® Gel Doc™ XR System; Bio-Rad Laboratories, Inc., Hercules, CA, USA). All experiments were repeated three times.

Immunohistochemical analysis. Cervical cancer samples were fixed in phosphate-buffered 10% formalin (pH 7.2), embedded in paraffin and cut into 4-µm sections. The sections were dewaxed in xylene, dehydrated in alcohol, and then incubated in 0.01 M sodium citrate buffer (pH 6.0) for antigen retrieval. Sections were incubated with 3% H₂O₂ for 30 min to block endogenous peroxidase activity, and with 10% milk at 37°C for 15 min to block non-specific binding of antibodies. Sections were then incubated with hWAPL, Oct4 or HPV E6 antibodies (1:100 dilution in PBS, cat. nos. sc-365189, sc-101534 and sc-460, respectively; Santa Cruz Biotechnology, Inc.) for 2 h at room temperature. This was followed by incubation with biotinylated secondary antibody (1:2,000, cat. no. sc-358919; Santa Cruz Biotechnology, Inc.) at room temperature for 1 h and visualization using 3,3'-diaminobenzidine under a light microscope.

Injection of transactivating transcriptional factor (TAT)-mediated si-hWAPL or si-HPV E6 into tumorspheres or exograft tumors in nude mice. Synthesis of TAT was performed as described in a previous study (16). In brief, 50 nM siRNAs (Ambion; Thermo Fisher Scientific, Inc.) were dissolved in RNase-free ddH₂O and then mixed with 10 µM TAT at a TAT:siRNA molar ratio of 20:1 and incubated for 30 min at 37°C. The siRNA sequences are shown in Table II. For *in vivo* use, treatment of the tumors was initiated at 2 weeks after tumorsphere implantation, as previously described (17). In brief, dissociated cells from tumorspheres were injected into the left and right sides of the mouse (as described above), and palpable tumors were formed 2 weeks later. At this time point, the tumor site on one side of the mouse was injected with TAT-mediated si-hWAPL (100 µM TAT/siRNA) and that

Table I. Primers for overexpression of hWAPL.

Gene	Primer	Sequence
hWAPL	Sense	5'-TTAAGCTTTGAAACTGGTGTCAAATGACATCCAGATT-3'
	Antisense	5'-TTGAATTCAAGCAATGTTCCAAATATTCAATCACTCTAGAG-3'
GAPDH	Sense	5'-AAGGCTGAGAATGGGAAAC-3'
	Antisense	5'-TTCAGGGACTTGTCTACTTC-3'

hWAPL, human wings apart-like.

Table II. siRNA sequences.

siRNA	Primer	Sequence (5'-3')	Product (bp)
Si-hWAPL 1	Sense	ACAGUUUUUAUCACUUUGGAU	-
	Antisense	CCAAAGUGAUAAAAACUGUGA	-
	Antisense	CCAGAUUUGGGAAAACAUACA	-
Si-HPV E6 1	Sense	GCAACAGUUACUGCGACGUUU	-
	Antisense	ACGUCUCGCAGUAAACUGUUGCUU	-
	Antisense	AGCUGGGUUUCUCUACGUGUU	-
Si-scrambled	Sense	GACCUGUUAUGACGGCACUU	-
	Antisense	GUGCCGUCAUUAACAGGUCUU	-
GAPDH	Sense	AAGGCTGAGAATGGGAAAC	254
	Antisense	TTCAGGGACTTGTCTACTTC	

siRNA, short interfering RNA; hWAPL, human wings apart-like; HPV, human papillomavirus.

on the other side was injected with control [scrambled (Scr) siRNA]. The tumor size was measured each week.

Cell invasion assay. Cell invasion was evaluated using 24-well Transwell® culture chambers, as previously described (17). Cells dissociated from tumorspheres were seeded at a density of 5×10^4 cells per well and cultured in stem cell medium (as mentioned above) containing 2% fetal bovine serum (cat. no. 10082139; Thermo Fisher Scientific, Inc.) for 24 h at 37°C in a humidified atmosphere containing 5% CO₂. Cells in the lower compartment were then fixed in methanol and stained with 5% crystal violet for 10 min at room temperature. The cells were counted under a light microscope. Three fields per sample were examined.

Statistical analysis. Data are shown as the mean \pm standard error of the mean. Comparisons of the groups were performed using analysis of variance, Fisher's exact test or two-tailed Student's t-test. $P < 0.05$ was considered to indicate a statistically significant difference.

Results

Expression of hWAPL and core stem cell TFs in undifferentiated CCSCs. All cervical cancer tissue specimens from the patients tested positive for HPV E6 expression, and the majority (15/17) were immunopositive for hWAPL (Fig. 1A).

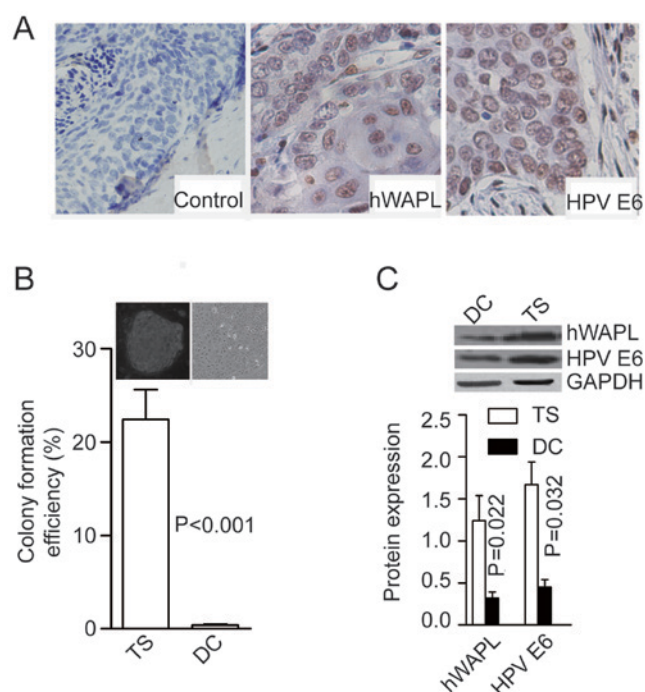


Figure 1. hWAPL expression in TS or DC. (A) *In situ* expression of hWAPL and HPV E6 in cervical carcinoma samples (magnification, $\times 400$). (B) Colony formation of cells dissociated from TS or DC ($n=3$). (C) Expression of hWAPL and HPV E6 in cells dissociated from TS or DC. GAPDH was used as the loading control. hWAPL, human wings apart-like; TS, tumorspheres; DC, differentiated cells; HPV, human papillomavirus.

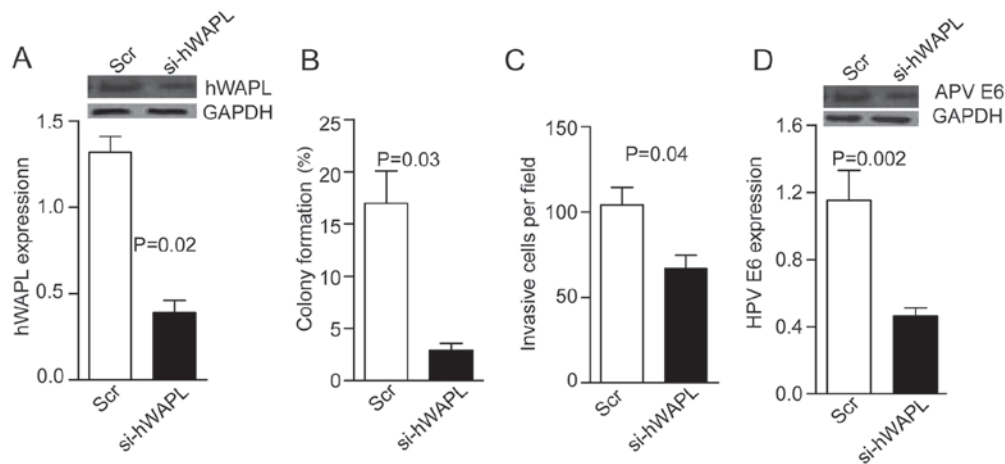


Figure 2. Knockdown of hWAPL induces tumorsphere differentiation and inhibits tumorsphere invasion and colony formation. (A) hWAPL expression, (B) colony formation, (C) invasion and (D) HPV E6 expression all decreased following hWAPL downregulation using si-hWAPL (n=3). hWAPL, human wings apart-like; HPV, human papillomavirus; si-hWAPL, short interfering RNA against hWAPL; Scr, scrambled control.

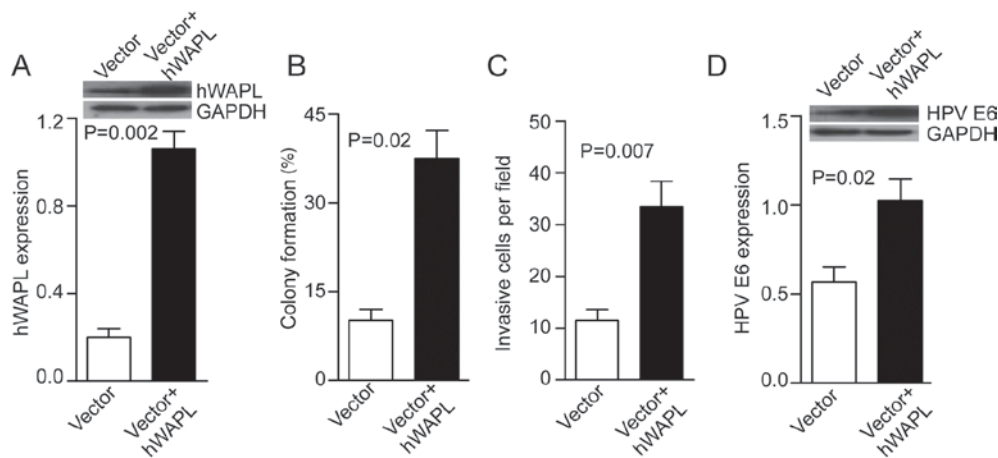


Figure 3. Overexpression of hWAPL maintains pluripotency and increases tumorsphere invasion and tumor formation. (A) hWAPL expression, (B) colony formation, (C) invasion and (D) HPV E6 expression all increased following the overexpression of hWAPL (n=3). hWAPL, human wings apart-like; HPV, human papillomavirus.

Following enzymatic dissociation and culture in stem cell medium, a few colonies (tumorspheres) formed in all of the tissues tested (17/17) after 2 weeks. Colonies were formed at the rate of $21.53 \pm 2.64\%$ for cells dissociated from tumorspheres, and only $0.41 \pm 0.07\%$ for cells dissociated from differentiated tumorspheres ($P < 0.001$; Fig. 1B). In addition, protein levels of hWAPL ($P = 0.022$) and HPV E6 ($P = 0.032$) were significantly higher in tumorspheres than in differentiated cells (Fig. 1C).

Knockdown of hWAPL induces tumorsphere differentiation and HPV E6 downregulation. The role of hWAPL in cervical cancer was investigated. TAT-mediated si-hWAPL was co-cultured with tumorspheres for 30 min. After 24 h, western blot analyses showed that hWAPL levels decreased by ~ 3 -fold in the si-hWAPL group compared with the controls ($P = 0.02$; Fig. 2A). Notably, in addition to decreased tumorsphere formation ($P = 0.03$; Fig. 2B) and invasion ($P = 0.04$; Fig. 2C), HPV E6 expression also decreased following the knockdown of hWAPL ($P = 0.002$; Fig. 2D).

Overexpression of hWAPL promotes tumorsphere tumorigenicity by increasing HPV E6 expression. Next, the tumorspheres were transfected with either Ad-hWAPL (Ad-hWAPL-GFP) vector for overexpression of hWAPL, or its control (Ad-scr-GFP). In the Ad-hWAPL transfected tumorspheres, hWAPL expression levels were increased 4-fold compared with those in the control ($P = 0.002$; Fig. 3A), colony formation ($P = 0.02$; Fig. 3B) and cell invasion ($P = 0.007$; Fig. 3C) were also increased. HPV E6 expression also increased following hWAPL overexpression ($P = 0.023$; Fig. 3D).

Knockdown of HPV E6 inhibits the invasion and colony formation of CTs. Following the coculture of TAT-mediated si-HPV E6 with tumorspheres for 30 min, the levels of HPV E6 were decreased ~ 4 -fold in the si-HPV E6 group compared with the control group ($P = 0.021$; Fig. 4A). HPV E6 knockdown inhibited hWAPL expression ($P = 0.024$; Fig. 4B), and also reduced colony formation ($P = 0.023$; Fig. 4C) and cell invasion ($P = 0.032$; Fig. 4D) compared with that of control CTs.

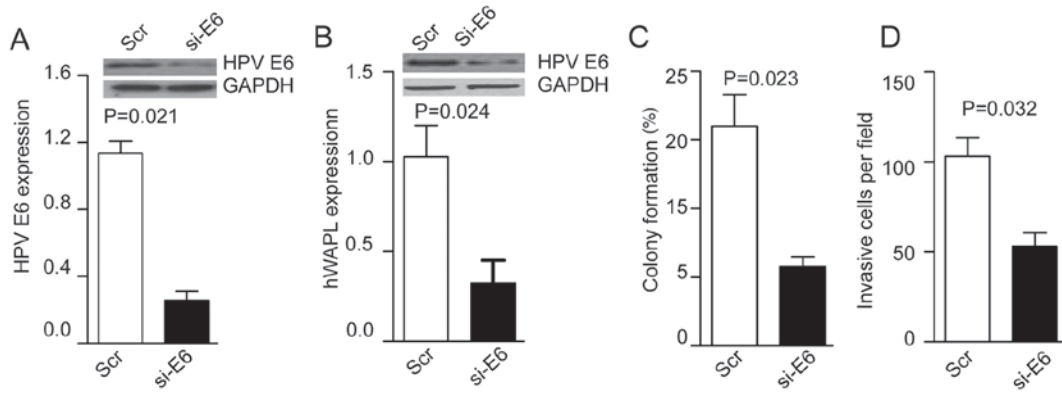


Figure 4. Knockdown of HPV E6 reduces hWAPL expression, colony formation and invasion in tumorspheres. (A) HPV E6 expression, (B) hWAPL expression, (C) colony formation and (D) invasion decreased in tumorspheres following HPV E6 knockdown using si-E6 (n=3). hWAPL, human wings apart-like; HPV, human papillomavirus; si-E6, short interfering RNA against HPV E6; Scr, scrambled control.

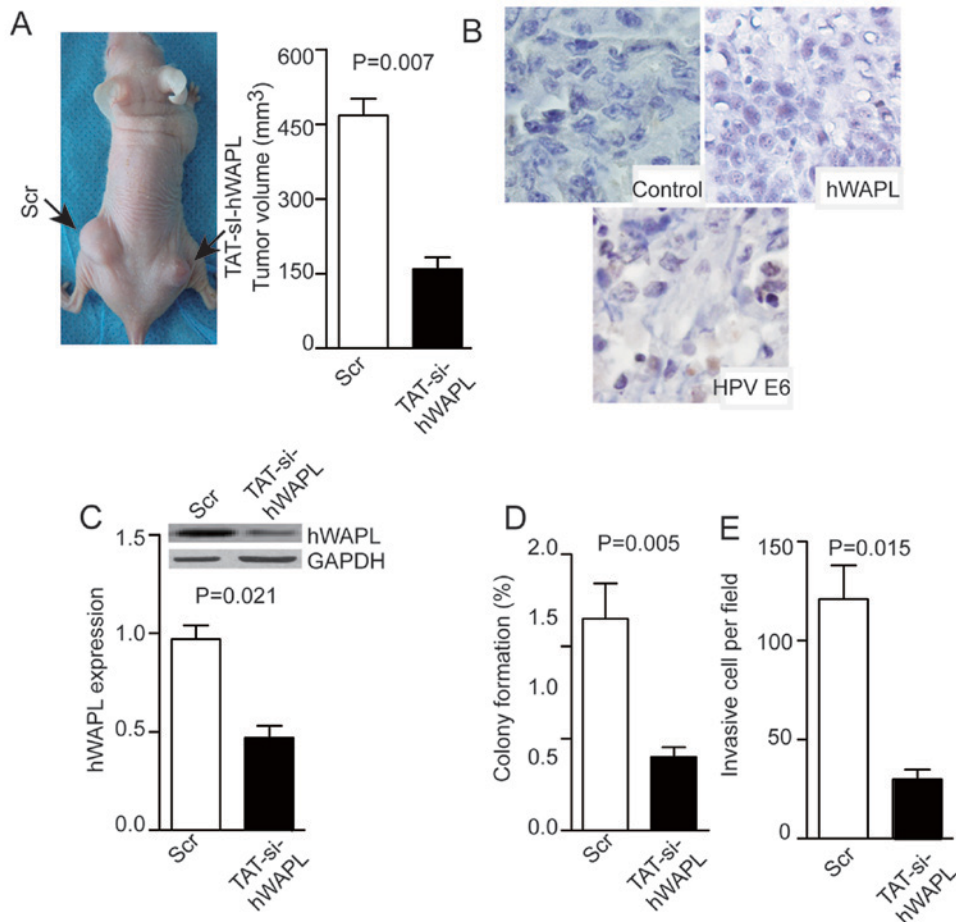


Figure 5. Injection of TAT-si-hWAPL into transplanted tumors inhibits tumor growth, hWAPL expression, colony formation, and tumorsphere invasion. (A) Injection of TAT-si-hWAPL into transplanted tumors inhibited tumor growth, (B) suppressed the expression of hWAPL and HPV E6 as shown by immunohistochemistry (magnification, x400), (C) decreased hWAPL expression as detected by western blot analysis and decreased (D) colony formation and (E) cell invasion (n=3). hWAPL, human wings apart-like; HPV, human papillomavirus; TAT-si-hWAPL, TAT-mediated short interfering RNA against hWAPL; Scr, scrambled control.

TAT-mediated si-hWAPL inhibits the growth of tumors derived from CTs. Tumor cells were dissociated from tumorspheres and injected into null mice. The resulting tumors were visible or palpable 2 weeks after injection. Subsequently, 100 μM/mg TAT/siRNA or its control (Scr) were injected into tumors once per week. Mice were sacrificed at day 28 and the

tumor volume was measured. Tumors in the TAT-mediated si-hWAPL group were smaller compared with those in the Scr group (P=0.007; Fig. 5A), and the weak expression of hWAPL and HPV E6 was detected after si-hWAPL treatment (Fig. 5B). The expression of hWAPL was decreased in the si-hWAPL group compared with that in the control group, as determined

by western blotting ($P=0.021$; Fig. 5C). Moreover, in the si-hWAPL group compared with the control group, colony formation ($P=0.005$; Fig. 5D) and cell invasion ($P=0.015$; Fig. 5E) was decreased.

Discussion

Cervical cancer is caused by HPV infection (18). The HPV E6 oncoprotein induces proteasome-dependent p53 degradation and inhibits expression of the p53 tumor suppressor protein. The HPV E6 protein targets the cellular E3 ubiquitin ligase E6AP to p53, resulting in the transfer of ubiquitin peptides from E6AP to p53, which induces degradation of p53 by the 26S proteasome (19). Knockdown of HPV E6 has been shown to efficiently kill HPV-positive cancer cells (20). In addition, it has been reported that HPV E6 is selectively overexpressed in CCSCs, and that the silencing of HPV E6 using siRNA abolishes CT formation and induces tumorsphere re-differentiation (21). In the present study, it was observed that HPV E6 was expressed in all the cervical cancer samples tested, indicating that it plays an important role in the maintenance of CCSC proliferation.

The WAPL gene was first identified in fruit flies (22,23). The human WAPL (hWAPL) gene is homologous in sequence to WAPL, is 30,793 base pairs in length, and is located on chromosome 10q23.2. The hWAPL gene encodes an aggregated anchored protein that disaggregates the polymerization of chromosome arms in the early stage of mitosis (24). The hWAPL protein is highly expressed in cervical cancer patients, and HPV E6 and E7 oncoproteins induce hWAPL expression (25). Moreover, HPV E6 is associated with cervical carcinogenesis, and as such, is a therapeutic target for cervical cancer (26). Nevertheless, little is known about the function of hWAPL in cervical cancer. The results of the present study indicate the potential of hWAPL as a marker of CCSC proliferation and suggest that hWAPL may play a role in maintaining the proliferation potential of CCSCs. It has previously been reported that HPV E6 induces hWAPL expression (25). In the present study, it was found that hWAPL has a counteractive effect on HPV E6 expression, indicating that HPV E6 and hWAPL interact in cervical carcinogenesis.

Cell-penetrating peptides (CPPs), which are short cationic polypeptides comprising ≤ 30 amino acids, have been used for the intracellular delivery of various macromolecules (27). TAT and MPG proteins from HIV-1, as well as penetratin and polyarginine, have been used as CPPs to facilitate the intracellular delivery of proteins and nucleic acids (28). TAT-CPPs enabled the safe and effective delivery of siRNAs for the knockdown of hWAPL in CCSCs to inhibit CCSC invasion and proliferation in the present study. In a previous study, Zhang *et al* (29) constructed a peptide that was able to deliver si-hWAPL to HeLa cervical cancer cells and successfully reduced hWAPL expression in those cells. As an extension of that previous study, the present study indicated that hWAPL interacts with HPV E6 and may be a marker of CCSC proliferation. Notably, the knockdown of hWAPL reduced cervical cancer proliferation, metastasis and recurrence. However, the present study has certain limitations. The experiments were only performed in mice, and should be repeated in higher level species. In addition, immune maintenance and whether

the local injection has any effect on other organs requires investigation in future studies.

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