

ALW-II-41-27, an EphA2 inhibitor, inhibits proliferation, migration and invasion of cervical cancer cells via inhibition of the RhoA/ROCK pathway

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Abstract. Recent studies have shown that the Eph receptor A2 (EphA2) and its inhibitor ALW-II-41-27 could regulate various cellular processes in several types of cancer. However, the manner in which ALW-II-41-27 affects the development of cervical cancer (CC) remains unknown. The present study aimed to evaluate the role of ALW-II-41-27 in inhibiting the proliferation, invasion and migration of human papilloma virus-positive CC cells and to verify whether Ras homolog family member A (RhoA)/Rho-associated protein kinase (ROCK) may be a crucial pathway involved in this process. Reverse transcription-quantitative PCR and western blotting analyses indicated an upregulation of EphA2 expression in CC cell lines (HeLa and CaSki). Furthermore, the results from MTT and colony formation assays indicated that ALW-II-41-27 inhibited cell proliferation. Results from wound healing and Transwell assays further demonstrated the inhibitory effect of ALW-II-41-27 on CaSki and HeLa cell migration and invasion, respectively. Furthermore, ALW-II-41-27 inhibited the protein expression of GTP-RhoA and ROCK1 in CaSki and HeLa cells. In addition, the ALW-II-41-27-induced inhibition of the biological function of CaSki and HeLa cells was promoted by cell co-culture with RhoA and ROCK inhibitors. Taken together, the present findings revealed that ALW-II-41-27 inhibited CC cell proliferation, migration and invasion by blocking the RhoA/ROCK pathway. These findings provide further insight into the mechanism of CC progression and

significant information for the development of potential therapeutic targets for CC.

Introduction

Cervical cancer (CC) is the second most common malignant tumor diagnosed in women worldwide (1,2). The recognition of human papillomavirus (HPV) as a primary cause of CC led to the development of HPV testing. There has been a shift from reflex HPV testing for mild cytological abnormalities, to co-testing with cytology and HPV, and lately to primary HPV screening (3). In the past decade, the popularization of CC screening has effectively improved the efficiency of diagnosis and treatment, while reducing the mortality rate. However, the number of new CC cases exhibits an annual increasing trend worldwide. In China, it is estimated that nearly 100,000 new diagnosed cases and >30,000 cases of CC-associated mortality occur each year (4). Advances in surgery, chemotherapy and postoperative radiotherapy have contributed to a 5-year survival rate of ~80%. However, the actual long-term outcome of clinical treatment is not satisfactory. The high metastatic rate of CC reduces this result to <50% (5,6). The clinical difficulties in CC treatment highlight the importance of identifying novel key biomarkers of CC, unraveling the pathogenetic mechanism of this disease and improving the treatment strategies.

The Eph receptor A2 (EphA2) is a transmembrane receptor tyrosine kinase, which has been shown to be elevated in several types of cancer. For example, it is positively correlated with the aggressive behavior of certain cancer cells, including malignant melanomas, breast cancer, pulmonary cancer, prostate cancer and esophageal cancer (6-11). These previous studies have reported high expression levels of EphA2 in both tissue and cell models of these diseases. This elevated expression level was shown to be associated with high histological grade, lymph node metastasis and poor prognosis in the aforementioned tumors (6-11). Upregulation of EphA2 and moderate or high expression of EphrinA-1 in squamous cervical carcinoma are associated with shorter overall survival, suggesting that they could be used as valuable prognostic markers (12). ALW-II-41-27 is a newly developed ATP-competitive

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EphA2 inhibitor (13). ALW-II-41-27 and small interfering RNA (siRNA)-based downregulation of EphA2 can effectively inhibit conditioned medium of cancer-associated fibroblast-induced gastric tumorigenesis (14). EphA2-siRNA inhibits proliferation and induces cell cycle arrest in SBC-5 cells by interfering with EphA2 gene expression (15). Similarly, ALW-II-41-27 and the tyrosine kinase inhibitor dasatinib inhibit the proliferation of EphA2-positive small cell lung cancer cells by downregulating EphA2 gene expression (15). Based on this evidence, the present study aimed to explore the mechanism by which ALW-II-41-27 may inhibit tumor progression in CC.

According to a recent study, EphA2 antagonism can notably inhibit lipopolysaccharide-induced Ras homolog family member A (RhoA)/Rho-associated protein kinase (ROCK) activation in lung tissues, whereas antagonism of EphA2 can be used as a therapeutic method for acute lung injury (16). In addition, G12 protein stimulation can promote CC invasion via RhoA/ROCK/JNK activation (17). Inhibition of triple functional domain protein expression markedly decreases the migratory and invasive abilities of CC cells and the expression levels of the RhoA/ROCK signal transduction factors [RhoA, ROCK and phosphorylated (p)-LIM kinase] that in turn contribute to cell migration and invasion (18). It has been demonstrated that human squamous CC tissues overexpress RhoA and ROCK-I/II, whereas RhoA promotes CC cell progression and tumor growth (19). RhoA/ROCK is therefore considered as an important pathway associated with EphA2-related CC development.

The present study aimed to explore the role of ALW-II-41-27 in human CC cells and to determine its underlying mechanism. The findings from this study may provide further insight into the mechanism of CC progression and offer a promising therapeutic target for the treatment of this disease

Materials and methods

Cell culture and treatment. The human CC HeLa, CaSki and C33A cell lines, and the non-cancerous ectocervical epithelial Ect1/E6E7 cell line, were purchased from The Cell Bank of the Type Culture Collection of The Chinese Academy of Sciences. These cell lines were maintained in RPMI-1640 medium (Gibco; Thermo Fisher Scientific, Inc.) supplemented with 10% FBS (Gibco; Thermo Fisher Scientific, Inc.) and 1% penicillin/streptomycin (Invitrogen; Thermo Fisher Scientific, Inc.) and placed at 37°C in a humidified incubator containing 5% CO₂.

The EphA2 inhibitor ALW-II-41-27 (MedChemExpress) was dissolved in sterile DMSO (10 mmol/l stock solution) and stored in aliquots at -20°C. CaSki and HeLa cells were treated with 200, 600 or 1,000 nM ALW-II-41-27 for 24, 48 or 72 h (20). The activation of the RhoA/ROCK pathway was achieved by pretreatment of these two cells with the RhoA/ROCK signaling agonist phorbol 12-myristate 13-acetate (PMA; 100 ng/ml; Sigma-Aldrich; Merck KGaA) for 30 min. The expression levels of EphA2, RhoA and ROCK were attenuated following treatment of these two cells with the RhoA inhibitor C3 transferase (5 µg/ml; Cytoskeleton Inc.) or ROCK blocker hydroxyfasudil (10 µM; Tocris Bioscience) for 2 h. These compounds were selected for cell pretreatment based on a previous study (21).

Reverse transcription-quantitative (RT-q)PCR. TRIzol® reagent (Invitrogen; Thermo Fisher Scientific, Inc.) was used for total RNA extraction from all types of cells according to standard procedures. The detection of EphA2 was performed via RT-qPCR using a high-capacity cDNA Reverse Transcription kit (Qiagen AB) and SYBR Premix Ex Taq (Qiagen AB). The conditions for cDNA synthesis were as follows: 42°C for 30 min and 85°C for 5 sec. The thermocycling conditions were as follows: 95°C for 3 min; 39 cycles of 95°C for 5 sec, 56°C for 10 sec, 72°C for 25 sec; 65°C for 5 sec; 95°C for 50 sec. The relative expression levels were normalized to endogenous control GAPDH and were expressed as 2^{-ΔΔC_q} (22). The sequences of the primers were as follows: EphA2 forward, 5'-CTGGTCTGCAAGGTGTCTGA-3' and reverse, 5'-TTG GACAACCTCCAGTAGGG-3'; and GAPDH forward, 5'-GAT ATTGTTGCCATCAATGAC-3' and reverse 5'-TTGATTTTG GAGGGATCTCG-3'.

Western blotting. All types of cells were separately lysed using RIPA lysis buffer (Sigma-Aldrich; Merck KGaA) and protein concentration was determined using the BCA method (Beyotime Institute of Biotechnology). Proteins (20 µg/lane) were separated by 10% SDS-PAGE and transferred onto PVDF membranes (MilliporeSigma). Membranes were blocked in 5% milk for 2 h at room temperature. The membranes were incubated at 4°C overnight with primary antibodies against EphA2 (cat. no. ab185156; 1:1,000; Abcam), GTP-RhoA (cat. no. 211164; 1:500; Abcam), ROCK1 (cat. no. 07-1458; 1:500; MilliporeSigma), total-RhoA (cat. no. ABS620; 1:400; MilliporeSigma) and GAPDH (cat. no. ab181602; 1:10,000; Abcam). Next, membranes were incubated with secondary anti-rabbit (cat. no. ab6721; 1:2,000; Abcam) or anti-mouse (cat. no. ab6728; 1:2,000; Abcam) antibodies at room temperature for 40 min. Enhanced chemiluminescence reagent (Pierce; Thermo Fisher Scientific, Inc.) was used to detect the signal on the membrane. The data were analyzed via densitometry using Image Lab™ version 3.0 software (Bio-Rad Laboratories, Inc.) and normalized to expression of the internal control (GAPDH).

MTT assay. CaSki and HeLa cells were harvested, seeded in 96-well plates (1x10³ cells/ml per well) and grown until 80% confluence. MTT solution (5 mg/ml; Sigma-Aldrich; Merck KGaA) was subsequently added to the cells (15 µl/well). Following 4 h of incubation of the cells with MTT solution at 37°C, DMSO was added (150 µl/well) to dissolve the formazan crystals. A microplate reader (Omega Bio-Tek, Inc.) was used for spectrophotometry-based measurements of the optical density at 490 nm.

Colony formation assay. CaSki and HeLa cells (7x10² cells/well) were seeded in a 6-well plate and cultured for 14 days to allow the formation of cell colonies. Following 14 days, the colonies were fixed with 10% formaldehyde for 30 min at room temperature and stained with 0.5% crystal violet for 5 min at room temperature. The images of the colonies (>50 cells) were obtained using a camera (Olympus Corporation) and counted manually.

Transwell assay. Transwell upper chambers (pore size, 8.0 µm; BD Biosciences) were precoated with Matrigel. A

total of 100 μ l CaSki and HeLa cell suspension (1×10^5 cells) in serum-free medium was separately added into the upper chamber. The lower chamber was filled with RPMI-1640 medium supplemented with 10% FBS. Following 20 h of incubation, non-invasive cells in the upper chamber were removed and cells that had invaded the lower chamber were stained using Giemsa solution at room temperature for 15 min. The number of invading cells was estimated using a light microscope (magnification, $\times 100$) and quantified using ImageJ version 1.8.0 software (National Institutes of Health). The relative invasion rate was calculated as follows: Number of invasive cells/number of inoculated cells $\times 100\%$. All procedures were repeated three times.

Wound healing assay. HeLa and CaSki cells (1×10^6 cells/well) were separately seeded in 6-well plates (Corning, Inc.) and cultured until they reached 95% confluence. The cell monolayer was slowly scratched with a sterile 200- μ l tip. Cellular debris was washed off with fresh medium and the remaining cells were cultured in serum-free RPMI-1640 medium accordingly. The images of the wound healing process were obtained at 0 and 24 h using a light microscope (magnification, $\times 100$). The area of wound was quantified using ImageJ version 1.8.0 software (National Institutes of Health). All procedures were repeated three times.

Bioinformatics analysis. The Gene Expression Profiling Interactive Analysis (GEPIA; <http://gepia.cancer-pku.cn/>) database is a website that provides the RNA sequencing expression data of 9,736 tumors and 8,587 normal samples from The Cancer Genome Atlas (TCGA) and the Genotype-Tissue Expression (GTEx) projects (23). This database was used to determine the expression difference of EphA2 between patients with cervical squamous cell carcinoma and endocervical adenocarcinoma (CESC) and healthy controls. A total of 306 tumor samples and 13 normal samples were obtained from GEPIA, with $|\log_2 FC| > 1$ and $P < 0.01$ as the cutoff; where FC is fold-change. The UALCAN (<http://ualcan.path.uab.edu/>) database is a portal for tumor subgroup gene expression and survival analyses, which provides data from TCGA, Clinical Proteomic Tumor Analysis Consortium (CPTAC) and The Children Brain Tumor Tissue Consortium (CBTTC) (24). This database was also used to determine the expression difference of EphA2 in patients with cervical squamous cell carcinoma and healthy controls. A total of 305 tumor samples and 3 normal samples were obtained from UALCAN. The analysis results are presented in the form of box plots.

Statistical analysis. The data are presented as the mean \pm standard deviation and were analyzed using GraphPad Prism version 8.0 (GraphPad Software, Inc.). One-way ANOVA followed by Tukey's post hoc test was used for data comparisons between multiple groups. $P < 0.05$ was considered to indicate a statistically significant difference. All experiments were performed at least three times.

Results

EphA2 expression is increased in CC cells. The result from GEPIA database analysis demonstrated that the expression

level of EphA2 in 306 patients with cervical squamous cell carcinoma and endocervical adenocarcinoma was significantly higher than that in 13 normal samples (Fig. 1A). The boxplot from the UALCAN database of 305 patients and 3 normal samples also demonstrated the higher expression of EphA2 in primary tumor samples compared with that in normal samples (Fig. 1B). Subsequently, RT-qPCR and western blotting were used to determine EphA2 expression in HPV-positive CC cell lines (CaSki and HeLa), in the HPV-negative CC cell line (C33A) and in immortalized human cervical squamous cells (Ect1/E6E7). The results indicated that EphA2 expression levels were significantly higher in C33A, CaSki and HeLa cells than those noted in Ect1/E6E7 cells. The upward trends in CaSki and HeLa cells were more obvious compared with that in C33A cells (Fig. 1C and D). Therefore, CaSki and HeLa cells were selected for the subsequent assays.

ALW-II-41-27 inhibits the proliferation of CC cells. To investigate the effects of ALW-II-41-27 on the progression of CC, the proliferative capacity of CaSki and HeLa cells was determined prior to and following treatment with different concentrations of ALW-II-41-27. The results from an MTT assay demonstrated that ALW-II-41-27 significantly decreased the proliferative capacity of CaSki and HeLa cells, especially when cells were treated for 48 and 72 h with 1,000 nM ALW-II-41-27 (Fig. 2A and B). In addition, the number of colonies of both CaSki and HeLa cells was significantly decreased following treatment with different concentrations of ALW-II-41-27 (Fig. 2C and D). In particular, the concentration of 1,000 nM exerted the most prominent inhibitory effect on cell colony formation (Fig. 2C and D). These results demonstrated that ALW-II-41-27 could inhibit CC cell proliferation.

ALW-II-41-27 inhibits the migration and invasion of CC cells. CC cell migration and invasion were detected prior to and following treatment with different concentrations of ALW-II-41-27. The results from the wound-healing assay indicated that ALW-II-41-27 significantly inhibited CaSki and HeLa cell migration (Fig. 3A and C). Furthermore, ALW-II-41-27 significantly inhibited CaSki and HeLa cell invasive activity (Fig. 3B and D). These findings also indicated a concentration-dependent inhibition of CC cell progression by ALW-II-41-27, with 1,000 nM ALW-II-41-27 exerting the most significant inhibitory effects. These findings indicated the inhibitory effect of ALW-II-41-27 on CC cell migration and invasion.

ALW-II-41-27 inhibits the RhoA/ROCK pathway in CC cells. It has been reported that overexpression of ROCK-I/II is associated with the progression of CC (19). A previous study confirmed that RhoA can promote the proliferation and migration of CC cells (19). These results indicate that ALW-II-41-27 suppresses the malignant activity of CC cells. A potential regulatory association between ALW-II-41-27 and the RhoA/ROCK pathway was therefore hypothesized. In the present study, western blotting analysis was performed to determine the expression of the proteins associated with the RhoA/ROCK pathway. The results indicated that the protein expression of GTP-RhoA and ROCK1 was significantly decreased by different concentrations of ALW-II-41-27 in

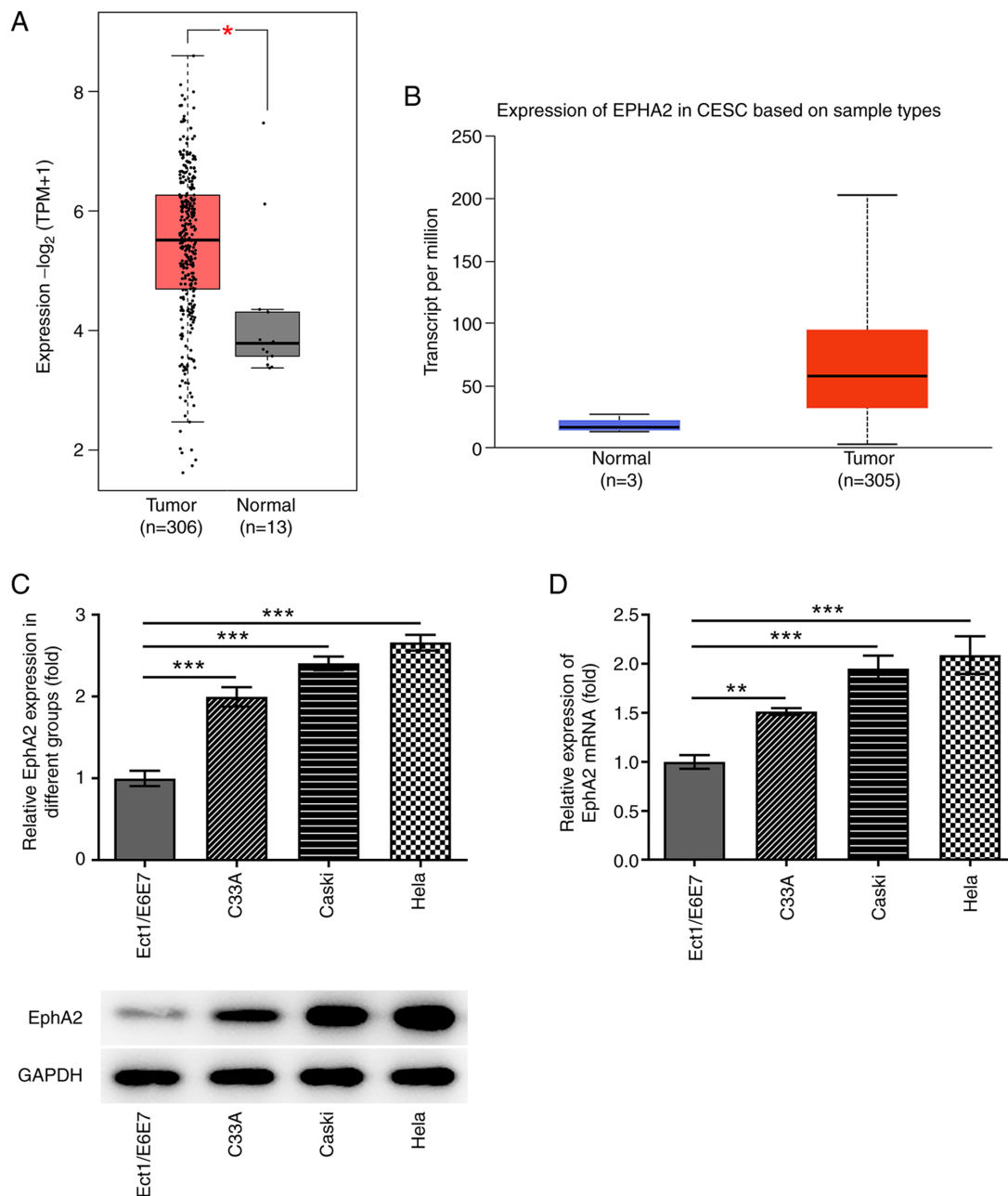


Figure 1. EphA2 expression is upregulated in cervical cancer cells. EphA2 expression in CESC and normal samples was examined using the (A) Gene Expression Profiling Interactive Analysis and (B) UALCAN databases. EphA2 expression was detected using (C) reverse transcription quantitative PCR and (D) western blotting. * $P < 0.05$, ** $P < 0.01$ and *** $P < 0.001$. EphA2, Eph receptor A2; CESC, cervical squamous cell carcinoma and endocervical adenocarcinoma; TCGA, The Cancer Genome Atlas.

CaSki and HeLa cells, whereas the total protein expression of RhoA was unchanged (Fig. 4A and B). In addition, ALW-II-41-27 at the concentration of 1,000 nM caused the highest downregulations of GTP-RhoA and ROCK.

ALW-II-41-27 inhibits the proliferation, invasion and migration of CC cells by blocking the RhoA/ROCK pathway. To explore whether ALW-II-41-27 could suppress CC cell progression via the RhoA/ROCK pathway, CaSki and HeLa cells were co-treated with ALW-II-41-27 and PMA in the absence or presence of C3 transferase or hydroxyfasudil. Since ALW-II-41-27 functioned in a concentration-dependent manner, the most efficient concentration of 1,000 nM was selected for the

following assays. The results from western blotting indicated that GTP-RhoA and ROCK1 protein expression was decreased following treatment with ALW-II-41-27 compared with that in the control group, while the addition of PMA largely reversed this change (Fig. 5A and B). Co-treatment of the cells with C3 transferase or hydroxyfasudil further decreased the expression of GTP-RhoA and ROCK compared with the expression following treatment with PMA + ALW-II-41-27, whereas that of total-RhoA level remained the same (Fig. 5A and B).

The results from the MTT assay revealed that ALW-II-41-27 significantly decreased CC cell proliferation compared with the control and that PMA hindered its effect. Co-treatment with PMA and C3 transferase or hydroxyfasudil significantly

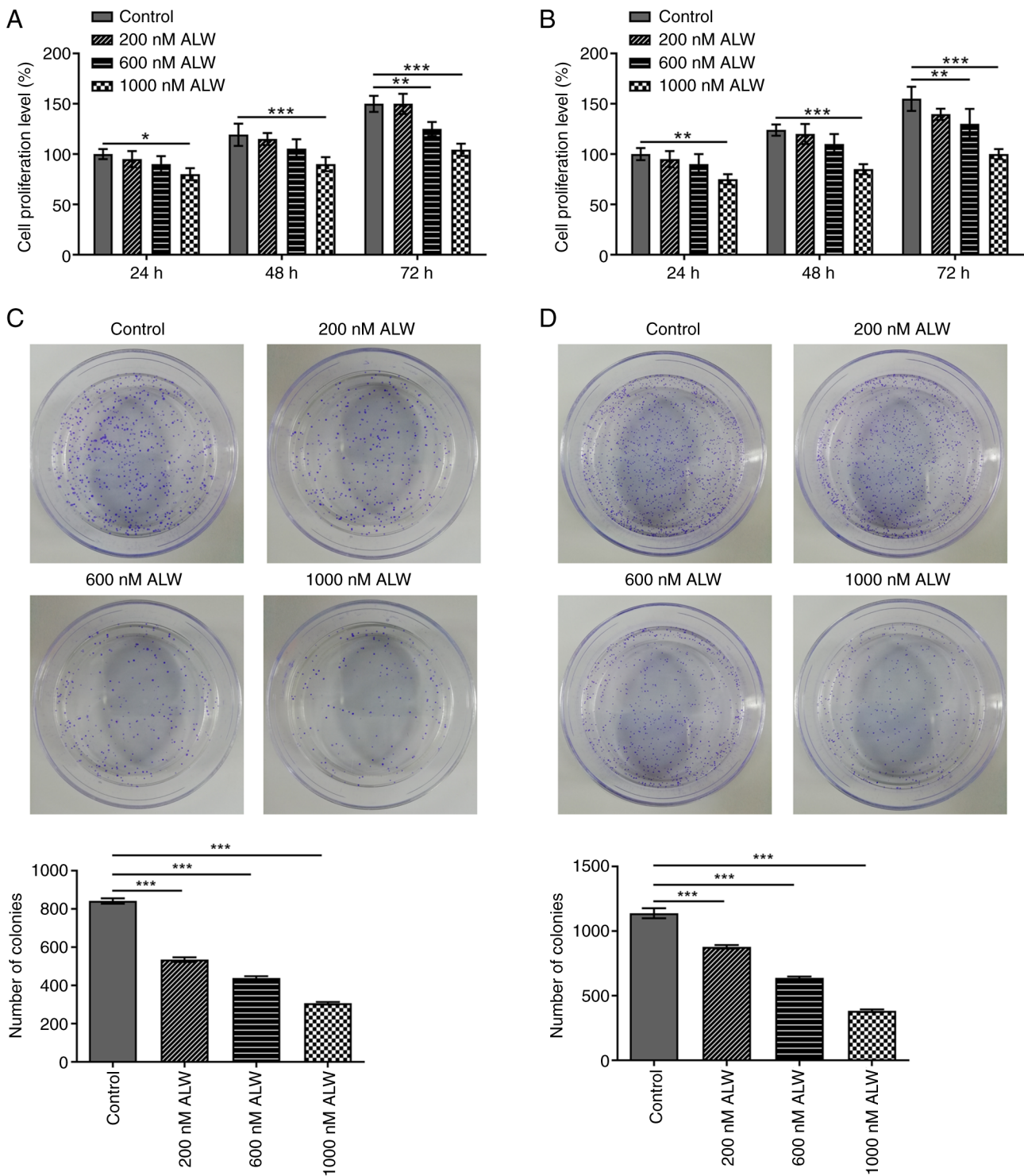


Figure 2. ALW-II-41-27 inhibits the proliferation of CaSki and HeLa cell lines. Proliferative activity was detected using the MTT assay in (A) CaSki and (B) HeLa cells treated with different concentrations of ALW-II-41-27 (200, 600 and 1,000 nM). Proliferative activity was detected using the colony formation assay in (C) CaSki and (D) HeLa cells treated with different concentrations of ALW-II-41-27. *P<0.05, **P<0.01 and ***P<0.001. ALW, ALW-II-41-27.

decreased ALW-II-41-27 + PMA-promoted cell proliferation at 72 h. CaSki and HeLa cells presented similar trends (Fig. 5C and D). The results of the colony formation assay indicated that co-treatment with PMA and C3 transferase or hydroxyfasudil inhibited cell colony formation, which was consistent with the results of the MTT assay (Fig. 5E and F).

The results from the wound healing and Transwell assays revealed that ALW-II-41-27-treated CaSki and HeLa cells

exhibited lower migratory and invasive activities compared with the control, while activities were partially restored in the PMA and ALW-II-41-27 co-treatment groups. The migratory activities of ALW-II-41-27-treated CC cells detected by wound healing assays were decreased following co-treatment with PMA and C3 transferase or hydroxyfasudil (Fig. 6A and C). The results of invasive activities from the Transwell assays presented similar trends among the different groups

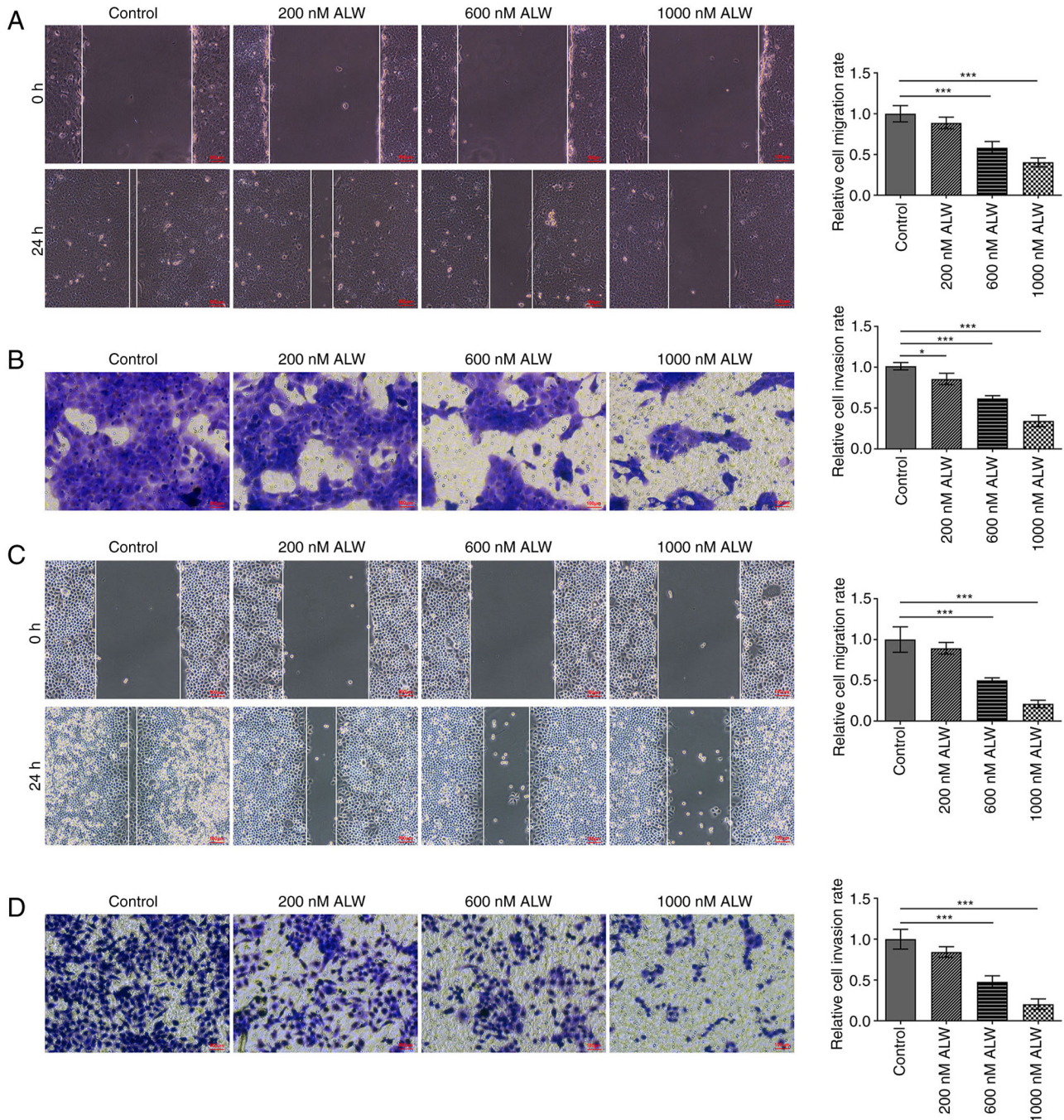


Figure 3. ALW-II-41-27 inhibits the migration and invasion of CaSki and HeLa cell lines. (A) Wound healing and (B) Transwell assays were used to assess the migratory and invasive activities of CaSki cells treated with different concentrations of ALW-II-41-27 (200, 600 and 1,000 nM). (C) Wound healing and (D) Transwell assays were used to assess the migratory and invasive activities of HeLa cells treated with different concentrations of ALW-II-41-27 (200, 600 and 1,000 nM). Magnification, x100. * $P < 0.05$ and *** $P < 0.001$. ALW, ALW-II-41-27.

(Fig. 6B and D). These findings indicated that ALW-II-41-27 could suppress the malignant activity of CC cells by blocking the RhoA/ROCK signaling pathway.

Discussion

CC ranks fourth among the most lethal gynecological malignancies worldwide (25). Despite the efforts made to improve the efficacy of standard treatment for CC, including surgical excision assisted by chemotherapy and radiotherapy (26,27), the 5-year overall survival rate remains low in metastatic

cases (28). Therefore, in-depth exploration and understanding of the pathogenesis and developmental process of CC are crucial for the development of novel therapeutic drugs and the search for novel treatment strategies.

EphA2 is expressed at high levels in several human tumors, such as prostate, bladder, skin and lung tumors (29). Furthermore, cancer cells of the highest tumor stage exhibit the highest expression levels of EphA2 (30). In addition, a previous study demonstrated that EphA2 overexpression was closely associated with a poor prognosis, a higher probability of metastasis and a shorter overall survival time in Ewing

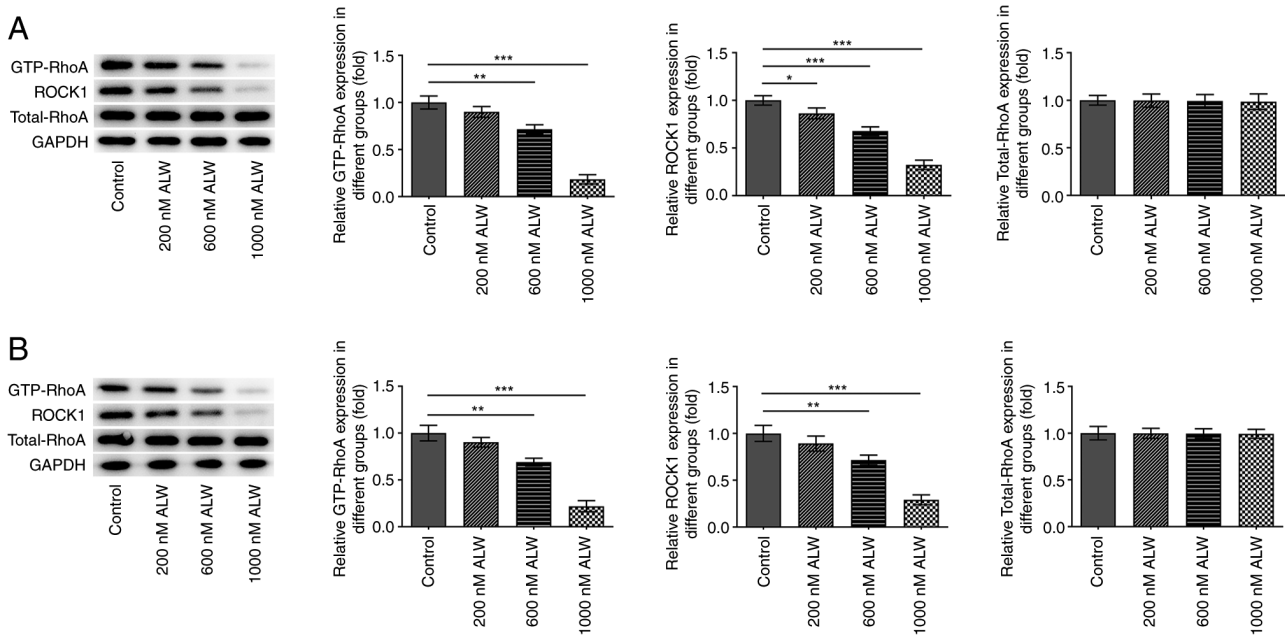


Figure 4. ALW-II-41-27 inhibits the expression levels of GTP-RhoA and ROCK1 in CaSki and HeLa cells. The expression levels of GTP-RhoA, ROCK1 and total-RhoA were determined via western blotting and RT-qPCR analyses in (A) CaSki and (B) HeLa cells following treatment with different concentrations of ALW-II-41-27 (200, 600 and 1,000 nM). * $P < 0.05$, ** $P < 0.01$ and *** $P < 0.001$. RhoA, Ras homolog family member A; ROCK1, Rho-associated protein kinase; RT-qPCR, reverse transcription quantitative PCR; ALW, ALW-II-41-27.

sarcoma (31). Furthermore, EphA2 plays a novel role in atherosclerosis, regulating both plaque inflammation and progression to advanced atherosclerotic lesions (32). EphA2 might also serve as a novel target for bladder cancer therapy (33). In addition, high expression of EphA2 together with EphrinA-1 is negatively correlated with the overall survival and prognosis of squamous cell CC (12). In the present study, EphA2 expression levels were assessed by western blotting and RT-qPCR analyses in CC cell lines. A significant increase in EphA2 expression was demonstrated in both HPV-positive CC cell lines and the HPV-negative CC cell line. Therefore, CaSki and HeLa cell lines were selected for subsequent experiments in the present study. To evaluate the function of EphA2 in the progression of CC cells, the EphA2 inhibitor ALW-II-41-27 was used to treat the CaSki and HeLa cell lines. The results from MTT and colony formation assays indicated that ALW-II-41-27 decreased the proliferative activity of the CaSki and HeLa cells in a concentration-dependent manner. Subsequently, Transwell and wound-healing assays were performed. The results demonstrated that ALW-II-41-27 could also inhibit the migratory and invasive activities of CaSki and HeLa cells, and that 1,000 nM ALW-II-41-27 exhibited an optimal inhibitory effect.

Investigation of the molecular mechanism of ALW-II-41-27 in inhibiting the growth and metastasis of CC was focused on its potential to regulate the RhoA/ROCK pathway. A previous study reported that the RhoA/ROCK pathway could mediate cardiomyocyte apoptosis via induction of oxidative stress and activation of the p38 MAPK and JNK pathways *in vitro*. These experiments were performed in primary cardiomyocytes isolated from ventricles of neonatal rats (34). In addition, it has been demonstrated that Shroom family member 2 inhibits tumor metastasis via the RhoA/ROCK

pathway in nasopharyngeal carcinoma (35). Targeting the RhoA/ROCK signaling pathway is also considered as an effective method to suppress breast cancer progression and lung cancer metastasis (36). Furthermore, a previous study reported that vascular endothelial growth factor-C facilitates CC metastasis via RhoA/ROCK-2-regulated activation of the moesin protein (37). Quercetin can induce apoptosis and loss of proliferation, migration and angiogenesis of SiHa CC cells by downregulating the expression levels of specific tumor-regulating genes, such as Akt1, Bcl-2-like 1, cyclin D1 gene, cyclin-dependent kinase 4, Polo-like kinase 1 and RhoA (38). In the present study, the protein expression of GTP-RhoA and ROCK1 was downregulated in a concentration-dependent manner following ALW-II-41-27 treatment, whereas the expression of total-RhoA did not change. Furthermore, to investigate the involvement of the RhoA/ROCK pathway in the effects of ALW-II-41-27 on CC cell progression, the RhoA/ROCK pathway agonist PMA, the RhoA inhibitor C3 transferase and the ROCK blocker hydroxyfasudil were used. Although PMA restored ALW-II-41-27-mediated inhibition of CC cell proliferation, migration and invasion, both C3 transferase and hydroxyfasudil could reverse the changes caused by PMA treatment. These results indicated that ALW-II-41-27 could inhibit CC cell proliferation, migration and invasion by inhibiting the RhoA/ROCK pathway. It is notable that the ROCK inhibitor Y-27632 has been shown to induce upregulation of p63 expression in CC cells (39). This protein is considered to play a fundamental role in cancer types arising from multi-layered epithelium, for example, p63 participates in the malignant phenotypes of esophageal squamous cell cancer cells (40,41). In addition, a previous study demonstrated that ROCK kinase could impede p63 nuclear transport in

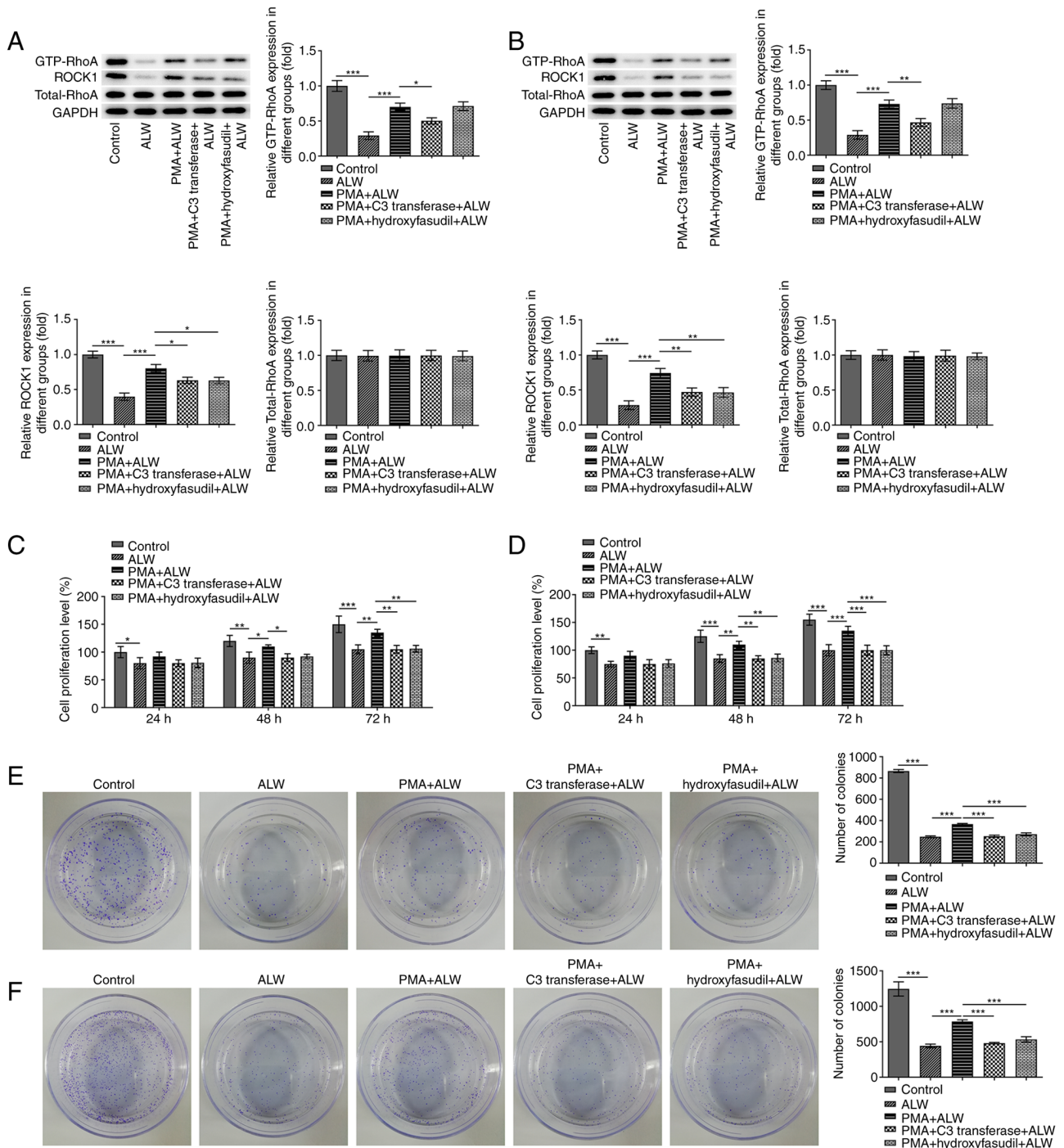


Figure 5. ALW-II-41-27 inhibits the proliferative, invasive and migratory activities of cervical cancer cells by blocking the RhoA/ROCK pathway. Expression levels of GTP-RhoA, ROCK1 and total-RhoA were determined by western blotting and reverse transcription quantitative PCR analyses in (A) CaSki and (B) HeLa cells treated with ALW-II-41-27, ALW-II-41-27 (1,000 nM) + PMA (100 ng/ml), ALW-II-41-27 + PMA + C3 transferase (5 μ g/ml) or hydroxyfasudil (10 μ M). The proliferation of (C) CaSki and (D) HeLa cells was assessed using the MTT assay. The proliferation of (E) CaSki and (F) HeLa cells was assessed using a colony formation assay. * $P < 0.05$, ** $P < 0.01$ and *** $P < 0.001$. RhoA, Ras homolog family member A; ROCK1, Rho-associated protein kinase; PMA, phorbol 12-myristate 13-acetate; ALW, ALW-II-41-27.

squamous cell carcinoma (42). The results from these two studies are fairly different compared with those from the aforementioned studies, as well as with the findings from the present study, suggesting that additional investigation is required to determine the exact underlying mechanism of ALW-II-41-27.

In summary, the present study demonstrated that EphA2 was expressed at abnormally high levels in CC cells.

Downregulation of EphA2 expression levels was induced following CC cell treatment with the inhibitor ALW-II-41-27, resulting in decreased cell proliferation, migration and invasion. ALW-II-41-27 may therefore impact the aforementioned aspects of CC cells, including proliferation, migration and invasion, by blocking the RhoA/ROCK pathway. The present study involved basic *in vitro* experiments. Additional in-depth mechanistic studies and *in vivo* experiments are therefore

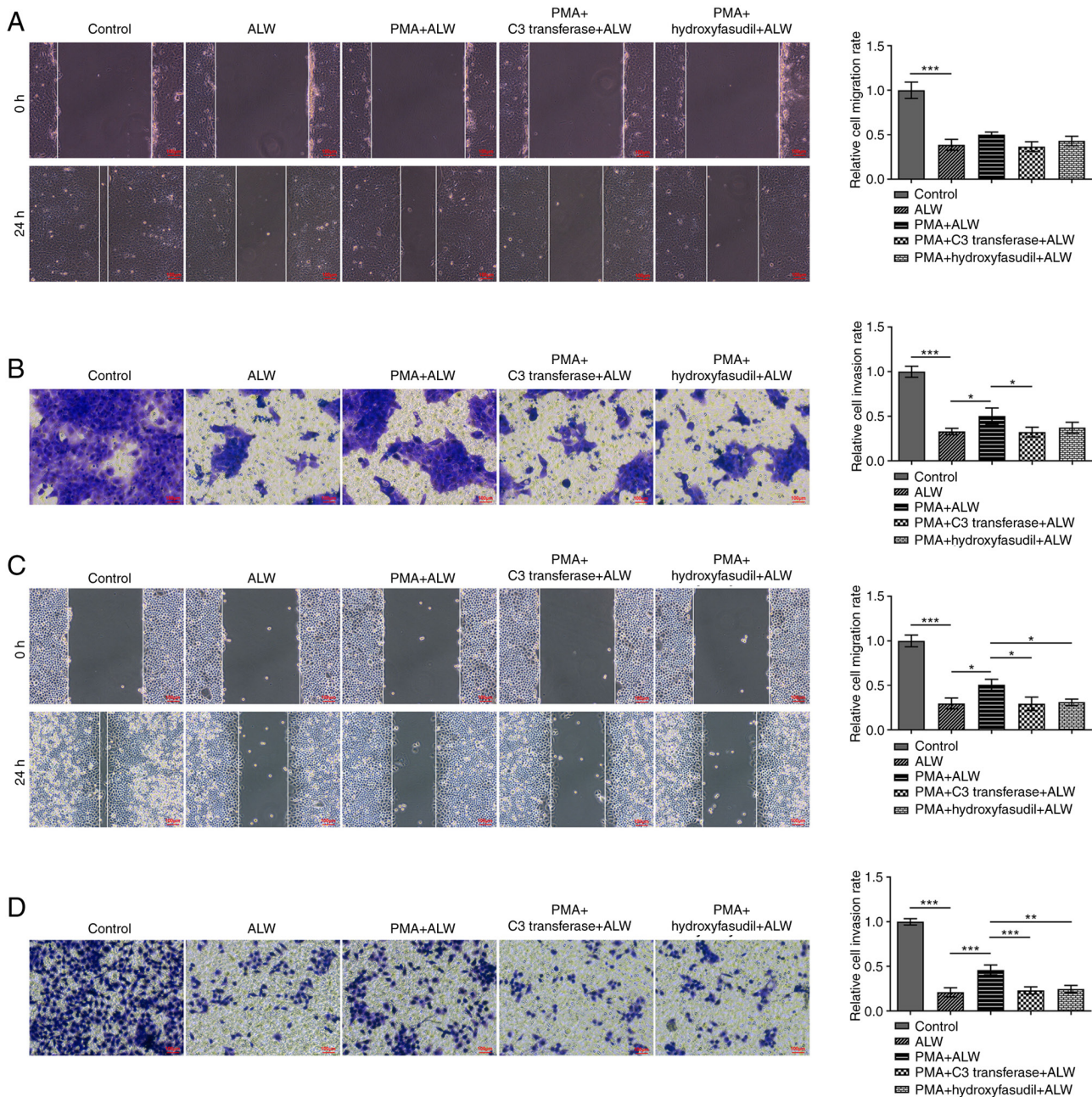


Figure 6. ALW-II-41-27 inhibits the proliferation, invasion and migration of cervical cancer cells by blocking the RhoA/ROCK pathway. (A) Transwell and (B) wound-healing assays were used to assess cell migratory and invasive activities of CaSki cells. (C) Transwell and (D) wound-healing assays were used to assess cell migratory and invasive activities of HeLa cells. Magnification, x100. * $P < 0.05$, ** $P < 0.01$ and *** $P < 0.001$. RhoA, Ras homolog family member A; ROCK1, Rho-associated protein kinase; PMA, phorbol 12-myristate 13-acetate; ALW, ALW-II-41-27.

required to confirm these findings. The present study explored the association between EphA2 and CC, and identified the crucial role of EphA2 in disease progression. Inhibition of EphA2 expression may thus be considered as a promising therapeutic strategy for the treatment or the control of CC. In addition, this study demonstrated that RhoA/ROCK may be a key pathway in the regulation of EphA2 and should therefore be examined further in the future to improve the understanding of CC pathogenesis.

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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

XL and DL contributed equally to this work and made substantial contributions to conception and design, acquisition of data

and analysis and interpretation of data. RM made substantial contributions to the conception and design of the study, and the analysis and interpretation of data, and was involved in drafting and critically revising the manuscript for important intellectual content. XL, DL and RM confirm the authenticity of the data, and have read and approved the final version of the 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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