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PACAP inhibits tumor growth and interferes with clusterin in cervical carcinomas

Ji-Hae Lee [a,b,1], Ji-Yeon Lee [a,b,1], Seung Bae Rho [b,1], Jong-Soon Choi [c], Dong-Gi Lee [c], Sungwhan An [d], Taejeong Oh [d], Don-Chan Choi [a], Seung-Hoon Lee [a,*]

[a] Department of Life Science, YongIn University, 470 Samga-dong Chuingu, Yonginsi, South Korea
[b] Research Institute, National Cancer Center, Ilsandong-gu, Goyang-si, Gyeonggi-do, South Korea
[c] Division of Life Science, Korea Basic Science Institute, Daejeon 305-806, South Korea
[d] Genomictree, Inc., 829 Tamnip-dong, Yuseong, Daejeon, South Korea

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ABSTRACT

Secretory clusterin (sCLU), an anti-apoptotic protein, is overexpressed in many tumors and enhances tumorigenesis and chemo-resistance. However, the regulation mechanism controlling the sCLU maturation process or activity remains undetermined. In this study, we found PACAP as a negative regulator of CLU. Overexpression of the PACAP gene in cervical cancer cell lines lacking PACAP expression significantly inhibited cell growth and induced apoptosis. We further demonstrated that interaction of PACAP with CLU significantly downregulated CLU expression and secretion, inhibited the Akt–Raf–ERK pathway, and suppressed the growth of human tumor xenografts in nude mice. This novel inhibitory function of PACAP may be applicable for developing novel molecular therapies for tumors with increased sCLU expression.

1. Introduction

Cancer is associated with the downregulation of tumor suppressor genes and upregulation of oncogenes. Gene expression can be regulated by genetic and epigenetic alterations; the former involves alterations in the DNA sequence, while the latter does not. DNA methylation is the most well-studied form of epigenetic alteration that leads to silencing of tumor suppressor genes, such as Rb, p16, MLH1, and BRCA1, resulting in the development and progression of cancer. Thus, the identification of abnormal gene methylation in cancer and characterizing the regulatory function of genes has implications in anticancer therapy [1]. In previous studies, hypermethylation of the PACAP gene was well established in cervical neoplasia [2], which suggests that epigenetic silencing by hypermethylation of PACAP leads to the loss of gene function and may play a critical role in human cervical tumorigenesis. PACAP is a novel peptide first isolated from ovine hypothalamus [3]. PACAP is a member of the vasoactive intestinal polypeptide (VIP)-glucagon-growth hormone releasing factor-secretin superfamily and has two amidated forms: PACAP-38 (38 amino acids in length) and PACAP-27 (a shorter form of the peptide with an 11 amino acid C-terminal cleavage) [4]. PACAP stimulates adenylate cyclase and subsequently increases cAMP formation in rat pituitary cells [3]. However, PACAP is also broadly expressed in the central nervous system and neuronal elements in the majority of peripheral organs.

In the human female genital tract, PACAP is located in nerve fibers with innervated blood vessels and smooth muscle cells of the internal cervical os [5,6]. PACAP and its receptor isoforms have been observed in the rat uterus, suggesting that PACAP regulates rat uterine function and physiology during the reproductive cycle [7]. PACAP either stimulates or inhibits the proliferation of cancer cells. In small lung cancer cells, prostate cancer cells, and neuroblastoma cells, PACAP stimulates cell proliferation [8–10]. In contrast, PACAP (6–38) inhibits breast cancer, lung cancer and prostate cancer growth [11–13]. Despite extensive studies, the molecular mechanism of tumor suppression by PACAP remains poorly understood.

To explore the function of PACAP, we identified PACAP-interacting proteins using the yeast two-hybrid system. We identified several genes and further characterized clusterin (CLU). CLU (also known as SGP2, ApoJ, CLI, or XIP8) is a ubiquitous secretory

* Corresponding author. Fax: +82 31 8020 2886.
E-mail address: shlee@yongin.ac.kr (S.-H. Lee).
[1] Contributed equally.

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heterodimeric glycoprotein with many biological functions, including lipid transportation, tissue remodeling, and regulation of apoptosis [14]. CLU has been reported as an anti-apoptotic and pro-apoptotic factor, and these ambiguous functions are caused by two different CLU isoforms, namely, sCLU and nCLU in various cell types [15–18]. sCLU is a general pro-survival factor in most cells after stress and is overexpressed in various human cancers such as prostate [19,20], breast [21], lung [22], renal [23], ovarian [24], colon [25], and cervical cancer [26,27]. In breast cancer, sCLU expression level is associated with large tumor size, and estrogen and progesterone receptor negative status, as well as with progression from primary carcinoma to metastatic carcinoma in lymph nodes [21]. In renal cell carcinoma, the expression level of sCLU was significantly correlated with pathological stage and the incidence of tumor recurrence [23]. In cervical cancer, CLU expression is significantly correlated with the paclitaxel IC_{50}, suggesting that CLU may antagonize the antitumor activity of paclitaxel [26]. Many previous reports have reported on the mechanisms of CLU in regulating cell survival and proliferation. However, the regulation mechanism controlling the sCLU maturation process or activity remains unclear. In this study, we examined the pro-apoptotic effect of PACAP in cervical cancer cells and propose that PACAP interferes with CLU-mediated cancer cell survival.

2. Materials and methods

2.1. Cell lines, mice, and transient transfection

Human cervical cancer cell lines were purchased from the American Type Culture Collection (ATCC; Manassas, VA) and maintained in recommended medium supplemented with 10% fetal bovine serum (JBI, Seoul, Korea) and 1% penicillin/streptomycin in a humidified 5% CO_2 incubator at 37 °C. Immune-deficient BALB/c nude mice were purchased from Orient Bio (Gyeonggi, Korea). Transient transfection was performed using the FuGENE HD transfection reagent (Promega, Madison, WI) according to the manufacturer's instructions.

2.2. Vector constructs

The construct pcDNA3-PACAP was obtained from Genomictree Inc. (Daejeon, Korea). CLU expression vector (pcDNA3.1-CLU) was obtained from the pIRES-CLU (full-length human clusterin) vector construct (provided by Dr. Saverio Bettuzzi, University of Parma, Italy). pIRES-CLU was digested with BamHI and NotI, and then inserted into pcDNA3.1. For yeast two-hybrid screening, PACAP was amplified by polymerase chain reaction (PCR) and inserted between the EcoRI and XhoI restriction sites of the pGilda vector. The primers used for amplification were forward, 5′-ATTAGAAT TCGGAATGACCATCTGTAGC-3′ and reverse, 5′-TAATCTCGAGTCG CTACAAATAAGCTAT-3′. For the β-galactosidase assay, CLU was inserted between the BamHI and NotI, restriction sites of the pGilda vector, and PCR-amplified PACAP was inserted into the pB42AD vector.

2.3. TUNEL assay

HeLa and HT-3 cells were seeded into four-well chamber slides and transiently transfected with pcDNA3-PACAP. After 48 h of transfection, TdT-mediated dUTP nick end labeling (TUNEL) assay was performed using the In Situ Cell Death Detection Kit (Roche Applied Science, Basel, Switzerland) in accordance with the manufacturer's instructions. Briefly, cells were washed with phosphate-buffered saline (PBS) and fixed in 4% paraformaldehyde (pH 7.4) for 1 h at room temperature followed by incubation in permeabilization solution (0.1% Triton X-100 and 0.1% sodium citrate) for 2 min on ice. The cells were then washed twice with PBS, and 50 μl of TUNEL reaction mixture was added to each sample and incubated in the dark in a humidified chamber for 60 min at 37 °C. The cells were washed three times with PBS and analyzed with a fluorescence microscope.

2.4. Cell growth assay

Transiently transfected HeLa and HT-3 cells grown in 24-well plates were trypsinized and harvested at 1-day intervals for 3 days. Cells were counted with a hemocytometer, and each experiment was repeated three times in triplicate wells.

2.5. CCK-8 cell proliferation assay

Cell proliferation was determined using Cell Counting Kit-8 (CCK-8) (Dojindo Laboratories, Kumamoto, Japan) according to manufacturer's instructions. Briefly, cells were seeded in a 96-well plate and transfected with PACAP, and absorbance at 450 nm was read on sequential days using microplate reader.

2.6. Protein extraction and Western blot analysis

Whole-cell lysates were prepared using RIPA buffer [50 mM Tris–Cl (pH 7.5). 150 mM NaCl, 1% NP-40, 0.5% sodium deoxycholate, 0.1% sodium dodecyl sulfate (SDS), 5 mM phenylmethanesulfonyl fluoride (PMSF)]. Conditioned medium was collected and separated from cell debris by centrifugation. Medium (1 ml) was concentrated by trichloroacetic acid (TCA) precipitation. The precipitate was dissolved in 100 μl of SDS sample buffer and 10 μl was loaded per well. Proteins (20–40 μg) were separated on an 8–12% SDS–polyacrylamide gel and transferred to a nitrocellulose membrane. The membrane was incubated with the appropriate primary antibody and horseradish peroxidase-conjugated secondary antibody. Antibodies against PACAP (sc-25439), CLU (sc-6419), Bax (sc-493), pERK (sc-7383), ERK (sc-94), GSK-3β (sc-8257), p-Raf (sc-12358) and GAPDH (sc-25778) were purchased from Santa Cruz Biotechnology. Antibodies against Akt (#4691) pAkt (#4060), pGSK-3β (#9331), pElk-1 (#9181) and cleaved PARP (#9541) were purchased from Cell signaling. Protein bands were visualized by enhanced chemiluminescence (ECL Western blotting detection regent; Bio-Rad) and quantified using the Image J program (NIH).

2.7. Yeast two-hybrid screening and β-galactosidase assay

Yeast two-hybrid screening was performed using the Matchmaker LexA two-hybrid system (Clontech, Mountain View, CA) according to the manufacturer's instructions. Briefly, PACAP cDNA was cloned into the pGilda bait vector as described above and human ovary cDNA library-cloned pB42AD prey vector. Bait and prey vectors were co-transformed in the EGY48 yeast strain using the lithium acetate method, and positive clones were selected on synthetic dropout medium lacking leucine, tryptophan, histidine, and uracil. Positive clones were further confirmed using the β-galactosidase assay with 5-bromo-4-chloro-3-indolyl-D-galactopyranoside (X-gal).

2.8. Co-immunoprecipitation (Co-IP)

Cells were lysed in NP-40 lysis buffer (20 mM Tris–HCl pH8.0, 150 mM NaCl, 1% Non-ident P-40, 1 mM PMSF) for 30 min on ice. Lysates were centrifuged at 13,000 rpm for 10 min at 4 °C, and the protein concentration was measured using the Bradford assay. Each cell lysate (1.5 mg) was incubated with Flag monoclonal

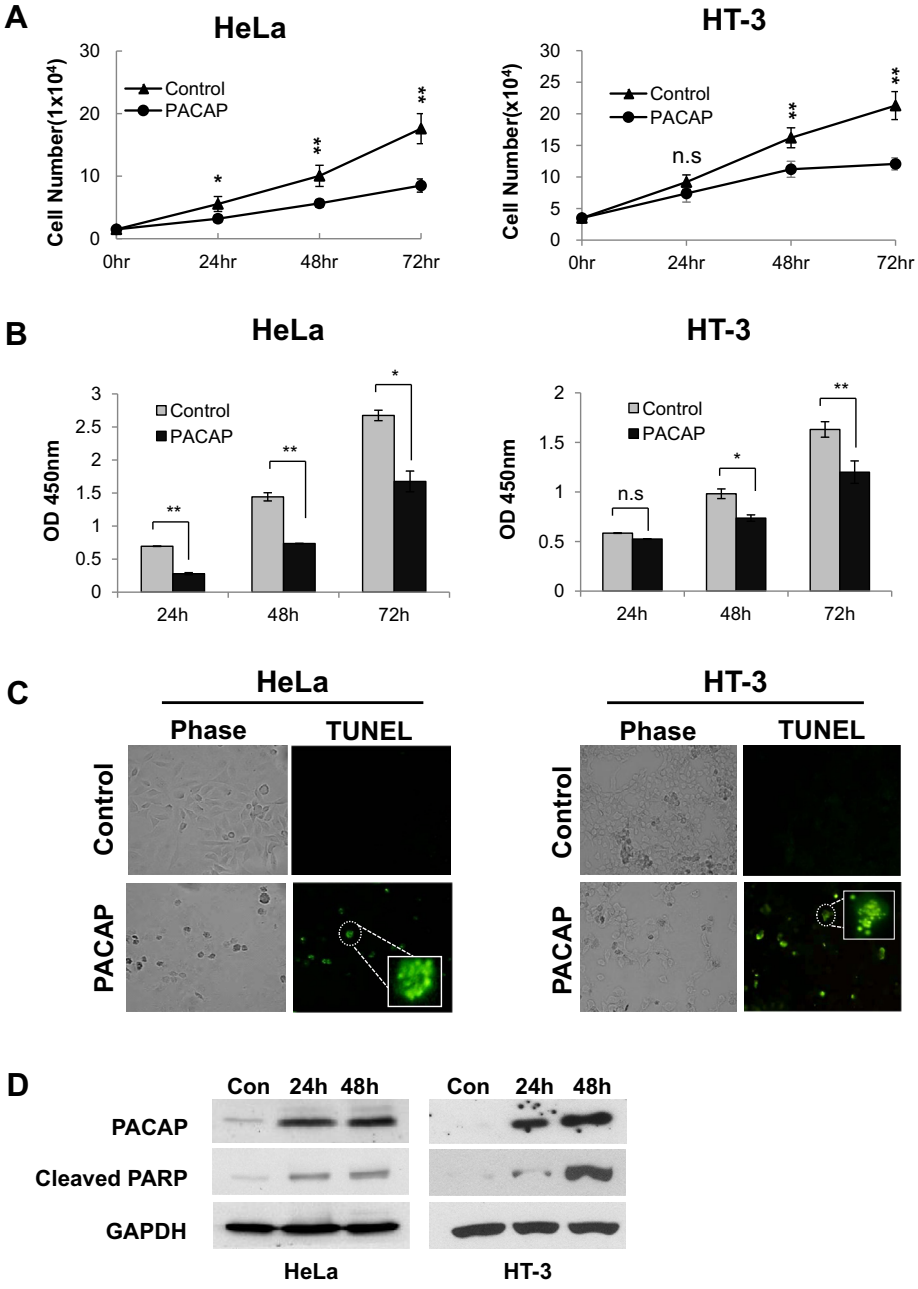

Fig. 1. PACAP inhibits cell growth and induces apoptosis in cervical cancer cells. (A) Cervical cancer cells, HeLa and HT-3, were plated in 24-well plates and transiently transfected with pcDNA3-PACAP. Cell numbers were counted at the indicated times. Error bars represent means ± S.D. $^{*}P < 0.05$, $^{**}P < 0.01$, n.s. not significant, compared with control. (B) Cells were transiently transfected with pcDNA3-PACAP and cell proliferation was measured by the CCK-8 assay. Error bars represent means ± S.D. $^{*}P < 0.05$, $^{**}P < 0.01$, n.s. not significant, compared with control. (C) HeLa and HT-3 cells were transiently transfected with pcDNA3-PACAP and TUNEL assay was performed 48 h after transfection. Images were captured on a fluorescence microscope. (D) Cells were transiently transfected with pcDNA3-PACAP, after which cells were harvested at 24-h intervals. The levels of PACAP and cleaved PARP were determined using Western blot analysis.

antibody (Sigma) for overnight at 4 °C. Following incubation, protein was immunoprecipitated using protein A/G agarose beads (Santa cruz) for 3 h at 4 °C with gently rotation. The immunoprecipitates was washed three times with lysis buffer and boiled in 40 μl of 1× SDS sample buffer for 5 min at 95 °C. After centrifugation, the supernatant was analyzed by Western blot.

2.9. Immunofluorescence analysis

HeLa cells were seeded onto 18-mm gelatin-coated glass coverslips and incubated for 24 h. Cells were then fixed and permeabilized with a methanol: acetone (1:1) mixture for 7 min at −20 °C. After blocking with 5% bovine serum albumin (BSA) for 1 h at room temperature, cells were incubated with anti-CLU or anti-PACAP antibodies for overnight at 4 °C. Cells were then washed with PBS and incubated with Alexa Fluor 488 goat anti-rabbit IgG (Green) and Alexa Fluor 568 donkey anti-goat IgG (Red) (Invitrogen) in darkness for 90 min at room temperature. Finally, cells were counterstained with 1 μg/ml DAPI for 1 min. Fluorescence was detected using confocal fluorescence microscopy.

2.10. Small interfering RNA transfection

The siRNA sequences used were as follows: PACAP siRNA, 5′-CCUAGGGAAGAGGUAUAAA3′, control siRNA, 5′-GGUGUGCUGUUUGGAGGUCTT-3′. As a negative control, we used siRNA targeting

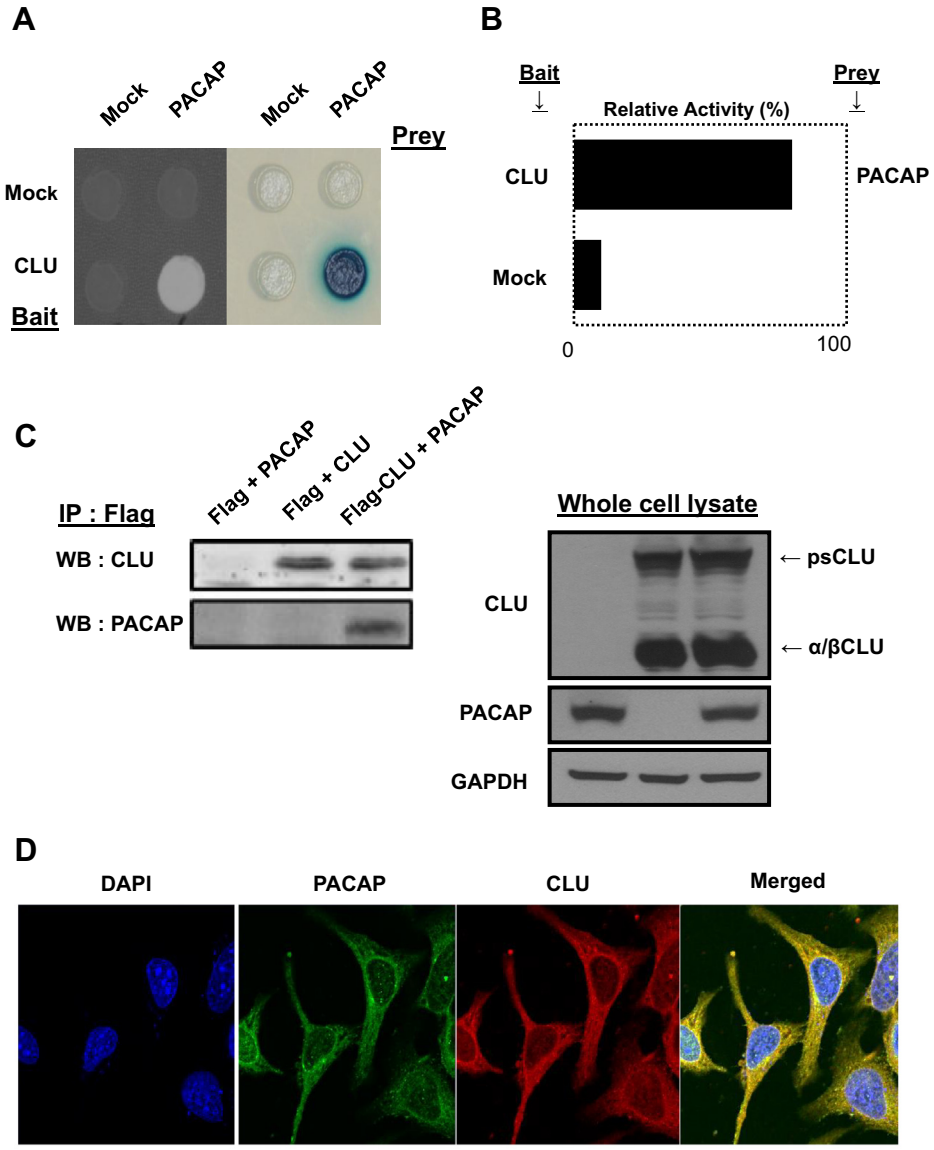

Fig. 2. PACAP interacts with CLU. (A) Transformants were assayed for their ability to grow on medium lacking leucine (left) and for β-galactoside expression (right). (B) Activity of the interaction between PACAP and CLU was represented by the activity of β-galactosidase expression. (C) Co-immunoprecipitation of PACAP with CLU. HEK293 cells were transfected with pcDNA3.1/Flag (control vector), pcDNA3.1/Flag-CLU and pcDNA3-PACAP. After 48 h, cell lysates were immunoprecipitated using anti-Flag antibody and immunoblotted using anti-CLU or -PACAP antibodies. (D) Cytoplasmic localization of PACAP and CLU. HeLa cells were seeded onto coverslips and incubated for 24 h. The endogeneous localization of PACAP and CLU were analyzed using confocal microscopy (magnification, ×400).

green fluorescence protein (GFP). Transfections were performed with Lipofectamine RNAiMAX Transfection Reagent (Life Technologies, Carlsbad, CA, USA) according to the manufacturer's instructions. The final oligonucleotide concentration was 30 nM.

2.11. s.c. tumor models

To establish tumors in mice, PACAP expressing pCDNA3 vector-transfected 1×10^6 HeLa cells were injected s.c. in the middorsal region of BALB/c nude mice (7–10 per group). Tumor size was evaluated using caliper measurements every 3 days. Mice were killed on day 33, and tumors were excised.

2.12. Statistical analysis

Data are presented as the mean ± S.D. For the analysis of statistical significance the student's *t* test (two-tailed) was used. Statistical significance representations: *, $P < 0.05$; **, $P < 0.01$.

3. Results

3.1. PACAP induces apoptosis in human cervical cancer cells

To explore the role of PACAP in human cervical cancer cells, PACAP overexpression was induced in HeLa and HT-3 cells. We first performed the cell counting and CCK-8 assay and found that overexpression of PACAP significantly reduced cell growth (Fig. 1A and B). To confirm that the reduction in cell growth represented apoptosis, we performed TUNEL assays and examined the morphology of nuclei in HeLa and HT-3 cells overexpressing control vector and full-length PACAP. PACAP-overexpressing cells showed fragmented nuclei characteristic of apoptosis, whereas control cells contained normal nuclei (Fig. 1C). To further examine apoptosis, cleaved PARP as a marker of apoptosis was detected using Western blot analysis. Full-length PACAP-overexpressing cells showed increased levels of cleaved PARP protein in a time-dependent manner (Fig. 1D). These results indicated that

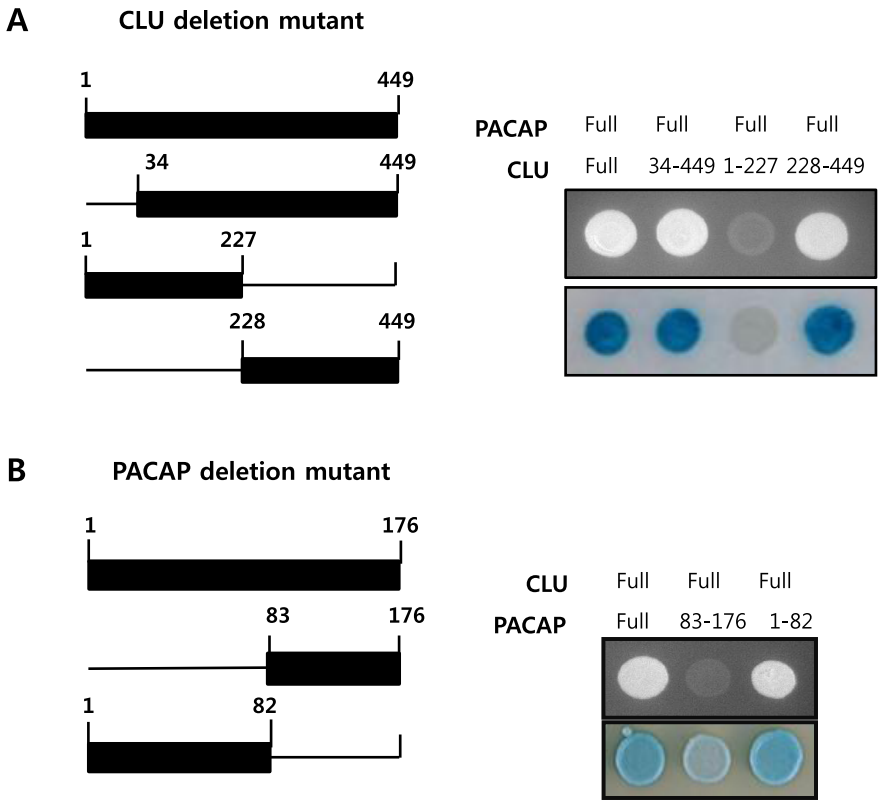

Fig. 3. Mapping of the interaction regions between PACAP and CLU. (A) Left panel displays the schematic diagram of cDNA constructs for each CLU deletion mutant, full-length CLU fusion proteins. Right panel exhibits the results of cell growth and β-galactosidase assays using yeast two-hybrid system. Positive interactions were revealed based on cell growth on leucine-depleted plates (upper panel), as well as the formation of blue colonies on the plate containing X-gal (lower panel). (B) Left panel displays the schematic diagram of cDNA constructs for each PACAP deletion mutant, full-length PACAP fusion proteins. Right panel shows the results of protein–protein interactions determined in the two-hybrid system.

overexpression of PACAP induced high levels of cell death in cervical cancer.

3.2. PACAP interacts and co-localizes with CLU

To further explore the function of PACAP, we screened the PACAP-binding proteins using the yeast two-hybrid system. We used pGilda-PACAP as bait to screen a human cDNA library. Based on these results, CLU was identified as an interacting partner. To confirm the interaction between PACAP and CLU, cell growth and β-galactosidase assays were performed (Fig. 2A and B). To examine the interaction between PACAP and CLU in human cells, HEK293 cells were cotransfected with Flag-CLU and/or pcDNA3-PACAP. An immunoprecipitation assay on cotransfected cells revealed strong interaction between PACAP and CLU (Fig. 2C). We next explored the subcellular localization of PACAP and CLU using immunocytochemistry analysis. Fig. 2D shows that both PACAP and CLU co-localized mainly in the cytoplasm.

3.3. Interaction domain between PACAP and CLU

To investigate the PACAP specific binding domain of CLU, we designed three CLU truncation fragments as shown in Fig. 3A. In the yeast two-hybrid system, full-length human PACAP cDNA and plasmid containing either full-length CLU cDNA (1–449) or three deletion mutant fragments (34–449, 1–227, or 228–449) were co-transformed into EGY48 yeast cells. Cells containing full-length, the 34–449 fragment (nCLU), or the 228–449 fragment (β-chain region) of CLU grew on the Ura, His, Trp, and Leu-deficient plates. However, the 1–227 cDNA fragment containing cells did not grow (right panel in Fig. 3A). To confirm these results, we determined the binding activity of these constructs by measuring the relative expression level of β-galactosidase. As shown in the right panel in Fig. 3A, β-galactosidase assay results confirmed that the mutant (1–227) could not bind to PACAP. These results suggested that PACAP binds to the β-chain of CLU. Subsequently, to determine the CLU binding domain of PACAP, we designed two PACAP deletion mutants (Fig. 3B). As shown in the right panel in Fig. 3B, full and 1–82 cDNA fragment containing cells grew on Ura, His, Trp, and Leu-deficient plates and X-gal containing plates. This result suggested that 1–82 region of PACAP that containing signal sequence (1–24) and pro-peptide (25–79) may interact with β-chain of CLU.

3.4. PACAP inhibits α/β CLU formation and secretion

To investigate the protein–protein interactions involved in the maturation of CLU to produce secretory CLU by PACAP, we analyzed CLU expression in intracellular and extracellular, whole cell lysate, and conditioned medium preparations from HeLa and HT-3 cells transfected with PACAP or CLU using Western blot analysis. Fig. 4A shows CLU expression in whole-cell lysate compared to the control, and CLU transfected and PACAP-transfected cells. Precursor CLU and α/β CLU was consistently expressed at high levels in the control. CLU (psCLU and α/βCLU) levels were elevated in full-length CLU-transfected cells. Transfection of PACAP effectively suppressed both precursor CLU and α/β CLU protein level. In addition, co-transfection with PACAP and CLU resulted in a reduction of CLU expression compared with CLU-transfected cells. To confirm the secretion of CLU, conditioned medium from HeLa and HT-3 cells overexpressing PACAP and full-length CLU was TCA-precipitated. Fig. 4B shows a protein band of 40kDa that corresponded to mature

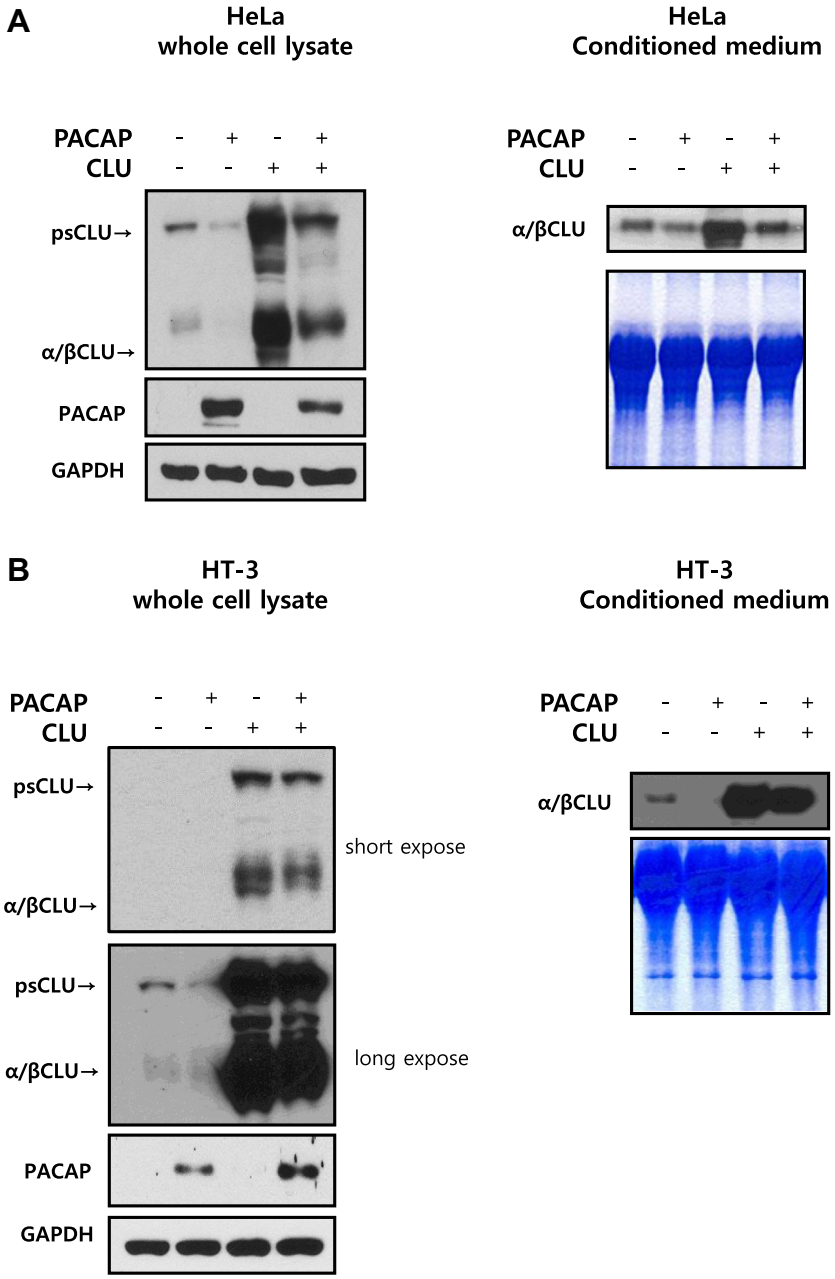

Fig. 4. Inhibition of CLU expression and secretion by PACAP. HeLa (A) and HT-3 (B) cells were transiently transfected with PACAP, CLU or PACAP plus CLU. Whole-cell lysates (left panel) and conditioned medium (right panel) were collected at 48 h after transfection. The Coomassie-stained gel is provided to show the equal amount of protein.

sCLU present in the conditioned media. Transfection of full-length CLU increased the secretion of mature sCLU. However, overexpression of PACAP significantly blocked sCLU secretion. These results suggested that formation of sCLU, the anti-apoptotic isoform of CLU, was inhibited by PACAP, and that the expression level in the intracellular and extracellular space was downregulated.

3.5. PACAP inhibits the Akt/Raf/Erk pathway

To explore the mechanism of growth inhibition and apoptosis induction by PACAP, we focused on the Akt pathway. Previous reports suggested that overproduction of sCLU results in Akt phosphorylation [28]. HeLa cells were transfected with PACAP for 24 or 48 h, and alterations in phosphorylated Akt were determined using Western blotting. As shown in Fig. 5, PACAP-overexpressing HeLa cells showed significantly reduced phosphorylation of Akt compared with control HeLa cells, which suggested that one of the major survival pathways activated by sCLU was suppressed by PACAP. We then analyzed the activation of Akt target proteins, which is affected by the overexpression of PACAP. As a result, the level of phosphorylated GSK-3β, Raf and ERK was markedly diminished in HeLa cells overexpressing PACAP. In addition, the phosphorylated form of Elk-1, the target of ERK also dramatically decreased. These data suggested that PACAP inhibits sCLU expression and secretion and inhibits HeLa cell growth by inhibiting the Akt/Raf/Erk pathway. CLU has been known as inhibitor of apoptosis by interfering with Bax pro-apoptotic activities. But PACAP did not effect on Bax expression.

3.6. PACAP-inhibited Akt/Raf/ERK pathway is mediated by CLU expression

To further confirm the inhibitory effect of PACAP on CLU expression and Akt/Raf/ERK pathway, we used RNA interference. As

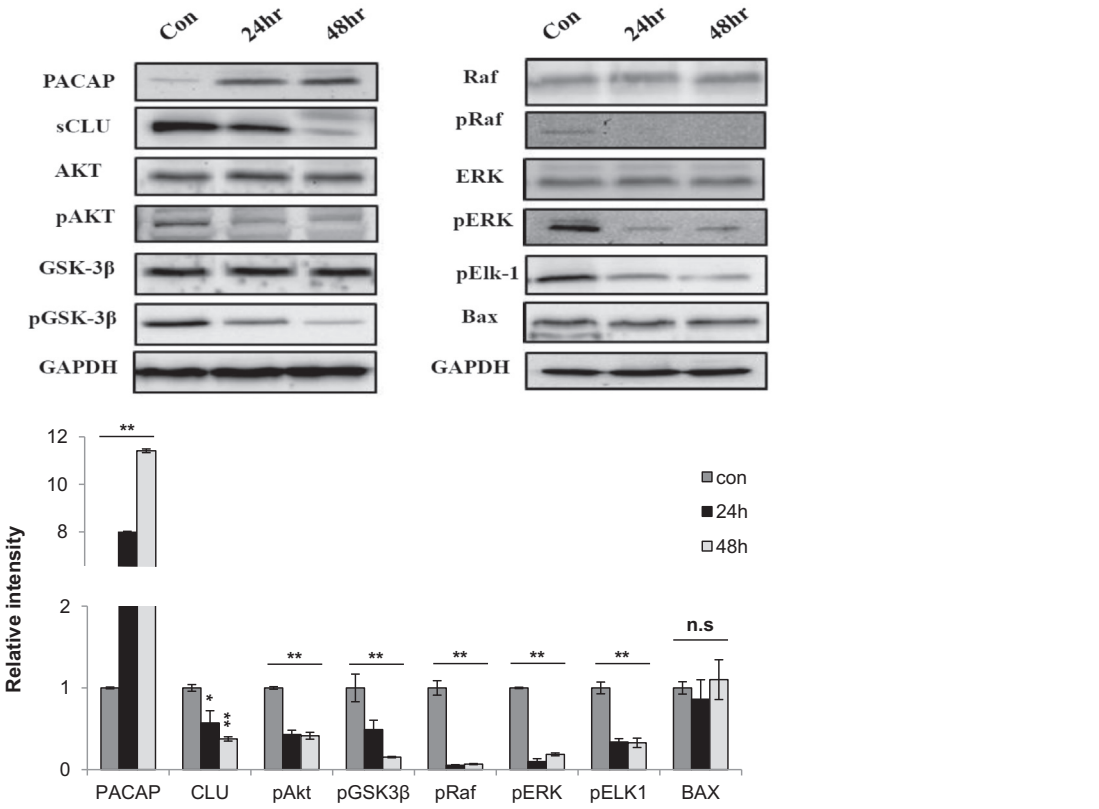

Fig. 5. Effect of PACAP overexpression on the Akt/Raf/ERK pathway. (A) HeLa cells were transiently transfected with PACAP, after which cells were harvested at 24 h intervals. Levels of Akt, pAkt, GSK-3β, pGSK-3β, Raf, pRaf, Erk, pErk, Elk-1 and Bax protein levels were measured by Western blot analysis. (B) The results of Western blot analysis were quantified by densitometry using the Image J program. Error bars represent means ± S.D. $^{*}P < 0.05$, $^{**}P < 0.01$, n.s. not significant, compared with control.

shown in Fig. 6A, siRNA markedly inhibited the expression of PACAP protein. And the inhibitory effect of PACAP on CLU-induced Akt and ERK activation was significantly restored by siRNA transfection (Fig. 6). From these results, we can conclude that the overexpression of PACAP causes inhibition of CLU expression, leading to Akt/Raf/ERK pathway, a key process for anti-proliferative activity and apoptosis.

3.7. PACAP inhibits tumor growth in nude mice

We next explored whether PACAP inhibits tumor growth *in vivo*. Exponentially growing HeLa cervical cancer cells were transiently transfected with the PACAP expression vector or control vector and the cells were injected subcutaneously into immune-deficient BALB/c nude mice. Animals (5–7 per group) were monitored for tumor growth. Tumor growth and morphology were analyzed over 30 days. Fig. 7 shows that the tumor mass in mice transfected with pCDNA3-PACAP was remarkably diminished compared to tumors transfected with the control vector. Collectively, these results demonstrated that PACAP can be a potent tumor suppressor in this animal model.

4. Discussion

Tumorigenesis is associated with activated or silenced gene expression, and gene expression is regulated by genetic or epigenetic alterations. DNA methylation is one of the best-studied epigenetic modifications that plays an important role in tumorigenesis.

Previous studies have reported that hypermethylation in the PACAP promoter is associated with low PACAP expression in cervical cancer cells [2]. The present study demonstrated that PACAP overexpression in cervical cancer cells significantly inhibited cell growth and induced apoptosis. Therefore, attenuation of PACAP expression may play important roles in the regulation of the cervical cancer development and progression. In this study, we observed a novel function of PACAP as a negative regulator of sCLU, an anti-apoptotic protein.

To further explore the function of PACAP, we screened PACAP-binding proteins and identified CLU, an enigmatic glycoprotein present in most human tissues and fluids [14]. The CLU gene generates at least two protein isoforms through alternative splicing: a secreted form (sCLU) and nuclear form (nCLU). The main product of CLU is an unglycosylated holoprotein (precursor secretory CLU, psCLU) with a predicted molecular mass of 60kDa. This psCLU is further glycosylated and proteolytically cleaved into α and β subunits held together by disulfide bonds. Mature CLU is secreted outside of the cells as a 76–80-kDa protein (sCLU) [29,30]. In contrast, nCLU lacks the leader peptide, which is an endoplasmic reticulum-targeting sequence. Thus, nCLU avoids cleavage and glycosylation and is detected as a 49-kDa precursor nCLU protein (pnCLU) in the cytoplasm. In response to stress [ionizing radiation, (TGF-β)], pnCLU is activated and translocated into the nucleus as a 55-kDa protein (nCLU) [15,16,31].

Previous studies have shown that overexpression of sCLU in cancer cells caused resistance to and protection against chemotherapeutic agents such as cisplatin, doxorubicin, etoposide, and camptothecin [32–34]. In addition, sCLU promotes hepatocellular carcinoma metastasis by enhancing cell motility and inducing the epithelial–mesenchymal transition process [35]. Niu et al. showed that sCLU silencing significantly inhibited tumor growth, motility, and invasion in breast cancer [36]. sCLU has chaperone-like activity in cytoprotection, scavenging denatured proteins and

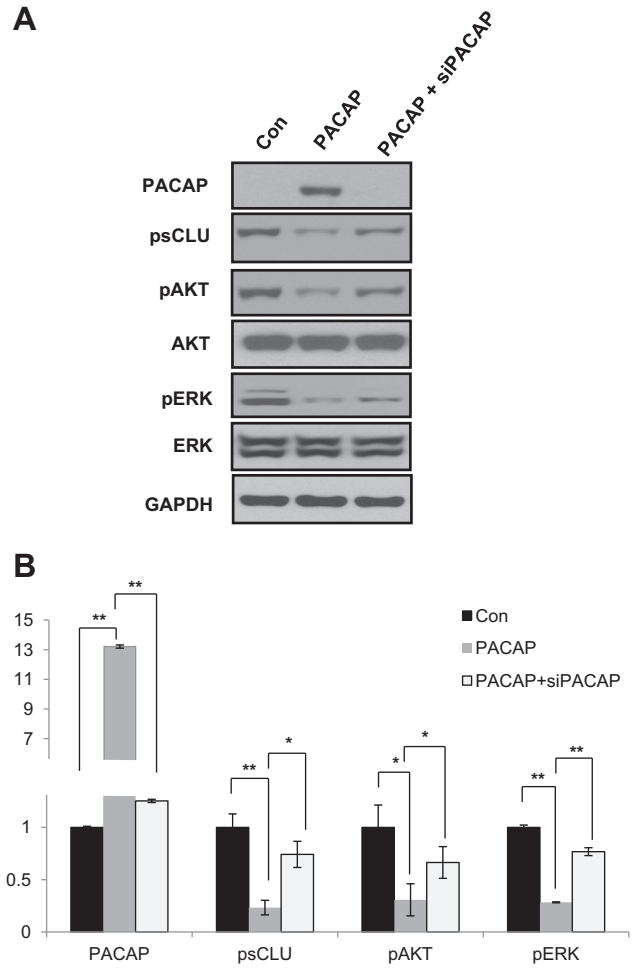

Fig. 6. PACAP knockdown recovers CLU expression and Akt-ERK phospholyration. (A) HeLa cells were transfected with either contorl-siRNA or PACAP-siRNA. After siRNA transfection for 24 h, the cells were transiently transfected with pcDNA-PACAP for 48 h. Levels of Akt, pAkt, Erk, pErk and CLU protein levels were measured by Western blot analysis. (B) The results of Western blot analysis were quantified by densitometry using the Image J program. Error bars represent means ± S.D. $^{*}P < 0.05$, $^{**}P < 0.01$, n.s. not significant, compared with control.

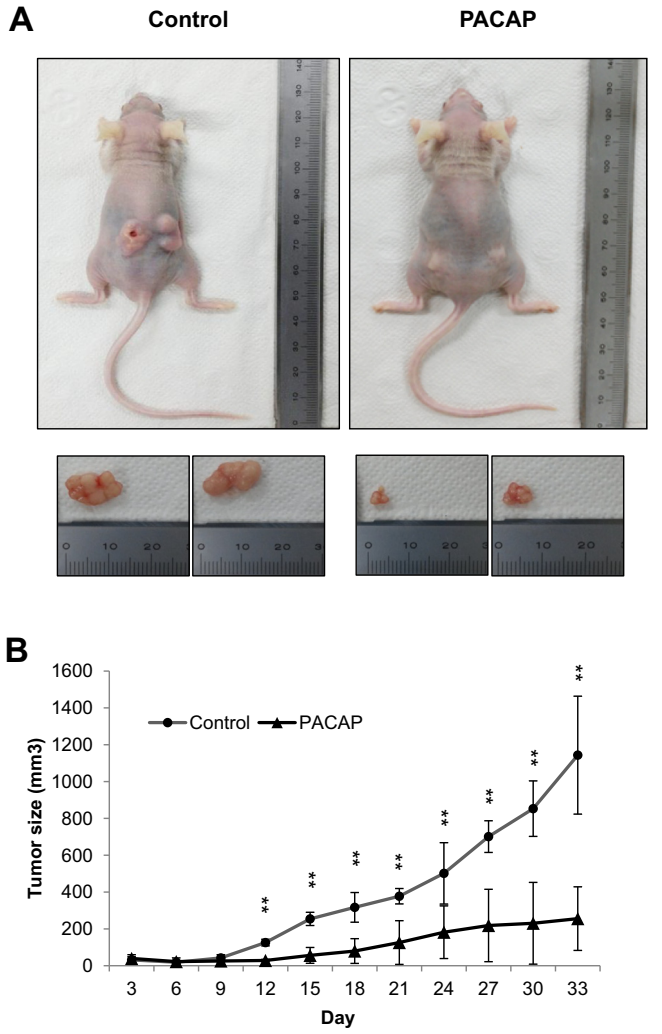

Fig. 7. Overexpression of PACAP suppresses tumor growth in vivo. HeLa cells were transiently transfected with the control vector and PACAP expressing vector and incubated for 24 h. Each of the 1×10^6 cells were inoculated by subcutaneous injection. (A) Image of tumor size at the time of dissection. (B) Tumor growth curve. Error bars represent means ± S.D. $^{**}P < 0.01$, compared with control.

cellular debris from outside the cells following specific stress-induced injury such as heat shock [37,38]. Our findings demonstrate that overexpression of PACAP inhibits tumor cell growth and induces apoptotic cell death (Fig. 1). Our yeast-two-hybrid assays and immunoprecipitation assay demonstrated that PACAP protein binds to CLU (Fig. 2). Overexpression of PACAP simultaneously inhibited the expression of precursor CLU and sCLU, as well as the secretion of sCLU out of the cell (Fig. 4). We speculate that PACAP overexpression affects various synthetic processes of CLU, which decreases sCLU precursor expression. In addition, a decrease in α/β CLU level and sCLU secretion suggests that the interaction of PACAP with CLU can inhibit the maturation of CLU to sCLU by blocking the cleavage of CLU to α and β. Our in vivo experiments also demonstrated that the overexpression of PACAP suppresses tumor growth in nude mice (Fig. 7). Thus, PACAP may be a target for gene therapy in sCLU-upregulated carcinomas by inhibiting sCLU expression and secretion, ultimately causing cancer cell death.

Recent studies have reported that the prostate cancer cell line MLL is protected from tumor necrosis factor-α (TNFα)-induced apoptosis by overexpressing sCLU, which is mediated in part by activation of the PI3K/Akt pathway. Overexpression of sCLU increased phosphorylation of Akt, activated Akt-induced phosphorylation of Bad, and decreased cytochrome *c* release from mitochondria, thus inhibiting TNFα-induced apoptosis. However, after inhibition of the PI3K/Akt pathway in sCLU inducible cells, approximately 40% of cells survived, indicating that another pathway may be involved in the survival effect of sCLU [28]. Zhong et al. [39] also demonstrated that once sCLU was overexpressed, Akt inhibition did not induce cell death, even in the presence of docetaxel. Akt plays important roles in mediating signals for cell growth, cell survival, cell-cycle progression, and differentiation. Akt has numerous downstream substrates, including Bad, pro-caspase-9, I-kB kinase, CREB, GSK-3, and Raf, which drive tumor progression [40]. Akt can directly phosphorylate Raf on S259, S364 on Raf-1, and S428 on B-Raf. Phosphorylated S259 by Akt can be inactivated by binding of the 14-3-3 protein [41]. However, inactivation of Raf by Akt may depend on the cell type and the stage of differentiation [42,43]. In addition, active Raf triggers the sequential activation of MEK and ERK. The CLU promoter contains three potential early growth response-1 (Egr-1) binding sites, and ERK transactivates the Egr-1 transcription factor required for sCLU expression [44]. We hypothesize that the interaction of PACAP with CLU inhibits the Akt activation function of sCLU and blocks the Akt pathway.

Consequently, we demonstrated that the Akt/Raf/ERK pathway was significantly inhibited in HeLa cells overexpressing PACAP (Fig. 5). We also observed decreased expression of sCLU in PACAP overexpressing cells were recovered by PACAP-siRNA. As expected, Akt and ERK phosphorylation was also increased by knockdown of PACAP (Fig. 6). Based on these results, the inhibited Akt/Raf/ERK pathway may be mediated by CLU in PACAP-overexpressing cells and it can be decreased cervical cancer cell growth.

Zhang et al. [34] reported that the α-chain of CLU interacts with Bax and that overexpression of the α-chain (but not the β-chain) of CLU suppresses Bax-induced apoptosis. Notably, we found that PACAP strongly interacted with the β-chain of CLU based on the yeast two-hybrid assay (Fig. 3). In addition, overexpression of PACAP showed no effect on Bax activity (Fig. 5). These results suggest that apoptotic cervical cancer cell death by PACAP does not occur through the Bax-mediated mitochondrial apoptotic pathway.

sCLU is a general anti-apoptotic factor in most cells, and recent clinical data suggest that aggressive tumors have elevated levels of sCLU with reduced nCLU expression. The ratio of sCLU to nCLU expression is a major determinant of cancer aggressiveness [45]. Currently, silencing sCLU expression using antisense oligonucleotides (ASO) or short-interfering double-stranded RNA has been developed and approved for clinical trials [46,47]. But there is no cellular molecules which can regulate CLU expression and the molecular mechanisms that control sCLU expression remain undefined. We propose PACAP as a first negative regulator of CLU. Since PACAP directly binds to CLU and interferes with CLU, it acts selectively in the specific inhibition of sCLU formation and secretion. Therefore, PACAP may have greater inhibitory effects on tumor cells expressing sCLU compared with normal cells. In conclusion, PACAP is an adenylate cyclase-stimulating protein that also blocks the cellular signaling cascades involved in tumor growth. This newly discovered mechanism can be used for the development of effective cancer therapies using PACAP.

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