Angiogenesis, Metastasis, and the Cellular Microenvironment

Atypical Protein Kinase \( \text{C}_i \) (PKCi) Promotes Metastasis of Esophageal Squamous Cell Carcinoma by Enhancing Resistance to Anoikis via PKCi-SKP2-AKT Pathway

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Abstract

Protein kinase \( \text{C}_i \) (PKCi) is an atypical PKC isoform and participates in multiple aspects of the transformed phenotype in human cancer cells. We previously reported that frequent amplification and overexpression of PKCi were correlated with lymph node metastasis in primary esophageal squamous cell carcinomas (ESCC). In the present study, short interfering RNA–mediated silencing of PKCi revealed that this enzyme was required for cell migration, invasion, and resistance to anoikis. In vivo experiments showed that PKCi suppression decreased tumor growth in esophageal cancer xenografts and lung metastases in nude mice. At the molecular level, knockdown of PKCi in suspended ESCC cells caused a decrease in S-phase kinase-associated protein 2 (SKP2) that had been reported to promote resistance to anoikis via the PI3K/AKT pathway. AKT phosphorylation was abolished after PKCi suppression, but AKT activation could be refreshed by PKCi upregulation, suggesting that PKCi enhanced cell resistance to anoikis via the PKCi-SKP2-PI3K/AKT pathway. Addition of the proteasome inhibitor MG132 prevented the decrease of SKP2 in PKCi silenced cells, and polyubiquitin-SKP2 was elevated after PKCi depletion, showing that PKCi might regulate the expression of SKP2 through the ubiquitin-proteasome pathway in suspended cells. Furthermore, overexpression of SKP2 in PKCi-downregulated cells restored cell resistance to anoikis. Most importantly, PKCi expression significantly correlated with SKP2 in 133 ESCC tissues (P = 0.031). Taken together, our data show that PKCi promotes tumorigenicity and metastasis of human esophageal cancer and that SKP2 is a candidate downstream effector of PKCi signaling in ESCC. Mol Cancer Res; 9(4); 390–402. ©2011 AACR.

Introduction

Esophageal squamous cell carcinoma (ESCC) is one of the most common human malignancies. The prognosis of ESCC patients has not significantly improved, with an average 5-year survival rate of 10% to 20% (1). Therefore, exploring the mechanisms involved in the development and progression of this disease is the key to effective treatment. The protein kinase C (PKC) family consists of at least 12 distinct lipid-regulated protein-serine/threonine kinases that are involved in multiple intracellular pathways and play pivotal roles in regulating cell proliferation, differentiation, and survival (2). The PKC isoenzymes are subdivided into 3 main classes: the cPKCs (conventional PKCs; PKC\(\alpha, \beta\)), the nPKCs (novel PKCs; PKC\(\delta, \epsilon, \eta, \) and \(\theta\)) and the aPKCs (atypical PKCs; PKC\(\zeta, \iota\), and \(\lambda\)). Atypical protein kinase C type 1 (PKCi) has been implicated in the establishment of cell polarity and cell survival (3). PKCi activity can be regulated by PI3K through PDK1 phosphorylation (4) and protein–protein interactions with upstream effectors, such as Ras (5). PKCi is reported to be amplified and overexpressed in several human cancers, and its overexpression is correlated with poor prognosis in lung, ovarian, and pancreatic cancer (6–10). We recently demonstrated that PKCi was frequently amplified and overexpressed in primary ESCC tumors. Moreover, the amplification and overexpression of PKCi were correlated with lymph node metastasis, which indicates that PKCi might play a role in ESCC progression (9). However, the molecular mechanisms underlying the role of PKCi in ESCC remain unclear.

S-phase kinase-associated protein 2 (SKP2) acts as a substrate-specific factor for the Skp1-Cul1-F-box (SCF) complex that regulates the destruction of multiple important proteins, including p27, p21, Rb, and p53 (11–13). Elevated SKP2 expression is frequently observed in many tumors, such as breast and prostate carcinomas (14, 15).
Our recent investigation revealed that SKP2 was amplified and overexpressed in primary ESCC tumors (16). Current studies have shown that Cdh1 is a critical E3 ligase that targets SKP2 for ubiquitylation and degradation (17, 18). However, the molecular mechanisms underlying elevated SKP2 expression have not been fully explored.

In the present study, we found that PKC\(_i\) promoted ESCC cell migration, invasion, resistance to anoikis, growth in soft agar, and tumor growth and lung metastasis in vivo. Mechanistically, we showed that PKC\(_i\) promoted resistance to anoikis through the SKP2-PI3K/AKT pathway and that PKC\(_i\) regulated the expression of anoikis-related SKP2 via the ubiquitin-proteasome pathway.

**Materials and Methods**

**Tissue specimens**

Fresh tissues containing ESCCs and adjacent histologically normal epithelia were procured from surgical resection specimens collected by the Department of Pathology in the Cancer Hospital, Chinese Academy of Medical Sciences, Beijing, China. Primary tumor regions and histologically normal esophageal mucosa from the same patients were separated by experienced pathologists and immediately stored at \(-70^\circ\)C until use. No treatment was received by any patients before surgery, and all patients signed separate informed consent forms for sample collection.

**Plasmid construction and small interfering RNA synthesis**

The full-length coding regions of human PKC\(_i\) and SKP2 were amplified from total cDNA of normal esophageal tissues using the following primers: PKC\(_i\)-forward: 5'-CCGGGATCC CGCCACCATGCCGACCCAGAGGGACACG-3' and reverse: 5'-CCGGGAATTCGACACATTC-3' and SKP2-forward: 5'-CCGGGAATTCGCCACCATGATGCACAGGAAGAGGACACGAGGACCTCGAG-3' and reverse: 5'-CCGGTCTCGAGTATGACACAGGACTTTGGTGTG-3'. The resultant PCR products were cloned into the pcDNA 3.1/myc-His A plasmid (Invitrogen). Correct construction was confirmed by DNA sequencing. Duplex PKC\(_i\) small interfering RNA (siRNA)-1 targeting 5'-AAAGCTGGAATATTTCTGGA-3' (2095-2113 in NM_002740 of PKC\(_i\); ref. 19), siRNA-2 targeting 5'-AACTTCTGGAAGACATGACCACAAGGTTACTAACCA-3' (2121-2148 in NM_002740 of PKC\(_i\); ref. 20) and a nonsilencing siRNA (5'-TTACTGGGACTTTCTGGA-3') were chemically synthesized (GeneChem) for transient transfection. PKC\(_i\)-forward: 5'-GATAGGTTGTGAGGTTTGGAGAC-3' and reverse: 5'-GATAGGTTGTGAGGTTTGGAGAC-3'. Glyceraldehyde 3 phosphate dehydrogenase (GAPDH) was used as a loading control. The PCR program was as follows: denaturation at 95\(^\circ\)C for 4 minutes; 30 cycles of 95\(^\circ\)C for 30 seconds, 58\(^\circ\)C for 30 seconds, and 72\(^\circ\)C for 40 seconds; followed by a 72\(^\circ\)C elongation step for 8 minutes; 10 \muL of each PCR product were resolved by 2% (w/v) agarose gel electrophoresis.

**Western blot analysis**

Proteins were separated by SDS-PAGE, transferred to polyvinylidene difluoride membranes (Millipore), blocked and probed with antibodies against PKC\(_i\) (1:2,000; BD PharMingen), \(\beta\)-actin (1:5,000; Sigma), AKT (1:1,000; Santa Cruz Biotechnology), and ubiquitin (1:500; Santa Cruz Biotechnology), and the primer sequences used for SKP2 were forward: 5'-GATGCTGCTTGCTTAGAATTGG-3' and reverse: 5'-GATGCTGCTTGCTTAGAATTGG-3'. Glyceraldehyde 3 phosphate dehydrogenase (GAPDH) was used as a loading control. The PCR program was as follows: denaturation at 95\(^\circ\)C for 4 minutes; 30 cycles of 95\(^\circ\)C for 30 seconds, 58\(^\circ\)C for 30 seconds, and 72\(^\circ\)C for 40 seconds; followed by a 72\(^\circ\)C elongation step for 8 minutes; 10 \muL of each PCR product were resolved by 2% (w/v) agarose gel electrophoresis.

**RNA extraction and reverse transcription PCR**

Total RNA was isolated using Trizol reagent (Life Technologies) according to the manufacturer’s instructions. Five micrograms of total RNA were used to synthesize the first strand of cDNA using 200 units/\muL SuperScript II RT (Invitrogen). The primer sequences used for PKC\(_i\) PCR were forward: 5'-GATGCTGCTTGCTTAGAATTGG-3' and reverse: 5'-TCACTGTGTGCCAGGTAAATTG-3'; and the primer sequences used for SKP2 were forward: 5'-CCTTTCTGGGTGTTCTGGATTCT-3' and reverse: 5'-GATAGGTTGTGAGGTTTGGAGAC-3'. Glyceraldehyde 3 phosphate dehydrogenase (GAPDH) was used as a loading control. The PCR program was as follows: denaturation at 95\(^\circ\)C for 4 minutes; 30 cycles of 95\(^\circ\)C for 30 seconds, 58\(^\circ\)C for 30 seconds, and 72\(^\circ\)C for 40 seconds; followed by a 72\(^\circ\)C elongation step for 8 minutes; 10 \muL of each PCR product were resolved by 2% (w/v) agarose gel electrophoresis.

**Haptotactic migration assays and Matrigel chemoinvasion assays**

For the haptotactic cell migration assay, 1 \times 10^5 PKC\(_i\) siRNA cells, nonsilencing siRNA cells, and parental EC9706 cells were seeded on a fibronectin-coated polycarbonate membrane insert in a Transwell apparatus (Costar). RPMI 1640 containing 20% FBS was added to the lower chamber. After incubation for 20 hours at 37\(^\circ\)C in a CO\(_2\) incubator, the insert was washed with PBS, and cells on the top surface of the insert were removed by wiping with a cotton swab. For the Matrigel chemoinvasion assay, the procedure was similar to the haptotactic cell migration assay, except that the Transwell membrane was coated with 300 ng/\muL Matrigel (BD Biosciences), and the cells were incubated for 36 hours at 37\(^\circ\)C. Cells that migrated to the
bottom surface of the insert were fixed with methanol, stained with 0.4% crystal violet and subjected to microscopic inspection. Cells were counted based on random 5 field digital images at ×200.

Adhesion assay
PKC\textsubscript{i} siRNA cells, nonsilencing siRNA cells, and parental EC9706 cells were plated on 100 ng/mL Matrigel-coated 96-well plates at a density of 5 \times 10^4 per well. The cells were incubated for 20, 40, 60, and 90 minutes at 37\degree C, respectively, in a CO\textsubscript{2} incubator. Nonattached cells were removed by 3 washings with PBS. Attached cells were analyzed using a 3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulfophenyl)-2H-tetrazolium (MTS; Promega) assay, according to the user’s manual.

Assessment of anoikis and treatment of inhibitor
Cells were cultured in dishes coated with PolyHEMA (Sigma), as described previously (22). Six-well polyHEMA plates were made by applying 1.5 mL of a 10 mg/mL solution of polyhydroxyethylmethacrylate in ethanol onto the plate and drying in the tissue culture hood. Cells were transiently transfected for 48 hours, trypsinized, and plated onto polyHEMA plates. Cells were harvested to assay for apoptosis using an Annexin V-FITC apoptosis detection kit (Sigma) and analyzed using flow cytometry after 24 hours of growth in suspension. Attached cells were treated for 24 hours with 20 \mu mol/L PI3K inhibitor LY294002 [2-(4-morpholinyl)-8-phenyl-4H-1-benzopyran-4-one; Calbiochem] at 24 hours posttransfection of PKC\textsubscript{i} siRNA. Detached cells were kept in suspension with 10 \mu mol/L of the proteasome inhibitor MG-132 (Sigma) for 24 hours.

Soft agar assay for colony formation
Colony formation assay in soft agar was based on a modified method (23). Cells transfected with PKC\textsubscript{i} RNAi or scramble sequence RNAi were plated in 6-well plates at a density of 500 cells per well. Three independent wells were examined per clone. The cells were incubated at 37\degree C in 5% CO\textsubscript{2}. After 3 weeks in culture, colonies were stained with 0.2% p-iodonitrotetrazolium violet (Sigma) and counted.

Xenograft assays in nude mice
Mixtures of stably transfected P4 and P8 or S1 and S2 clones were injected into 4- to 6-week-old female athymic nu/nu nude mice (Vital River). For each group, 6 mice were injected subcutaneously (s.c.) with 1 \times 10^6 cells per animal. The mice were sacrificed 4 weeks after injection and examined for s.c. tumor growth. Immunohistochemistry was performed on 5-\mu m sections of paraffin-embedded s.c. tumors. The slides were deparaffinized, rehydrated and dipped in a 3% hydrogen peroxide solution for 10 min, heated in citrate buffer (pH 6.0) for 25 min at 95\degree C, cooled for 60 minutes at room temperature, blocked by normal goat serum for 30 minutes at 37\degree C and then incubated with anti-PKC\textsubscript{i} antibody (1:100, BD PharMingen) for 2 hours at 37\degree C. After washing with PBS, the slides were incubated with biotinylated secondary antibody (1:100) for 30 minutes at 37\degree C followed by streptavidin--peroxidase (1:100). The development of the slides was performed using a diaminobenzidin solution. Counterstaining was done with hematoxylin. Eight mice were tail vein injected with 1.5 \times 10^6 cells per animal. The mice were killed 11 weeks after injection and examined for metastasis nodules in the lung. The tissues were fixed in Bouin’s solution, embedded in paraffin, cut into 5-\mu m sections, and stained with hematoxylin and eosin (H&E).

In vivo ubiquitination assay
Cells on 10-cm plates were transfected with PKC\textsubscript{i} siRNA-1 or nonsilencing siRNA. After being transiently transfected for 24 hours, cells were trypsinized, plated onto polyHEMA plates, and treated with MG132 for 24 hours. Then cells from each plate were collected into 2 aliquots. One aliquot (3 mg of cell lysate) was used for each immunoprecipitation reaction with 5 \mu g SKP2 antibody. Immunoaccepts were pulled down as described previously (24), resolved by SDS-PAGE, and immunoblotted for the presence of the polyubiquitin-SKP2 protein complex using anti-ubiquitin antibody. Another aliquot was used for conventional Western blotting to confirm PKC\textsubscript{i} expression knockdown and for a loading control.

Tissue microarray and immunohistochemical staining
A total of 133 formalin-fixed, paraffin-embedded ESCCs and corresponding normal epithelia were placed on the tissue microarray. For each case, normal tissues were repeated 3 times and cancer tissues were repeated 5 times. Immunohistochemical analysis was performed as mentioned before. PKC\textsubscript{i} and SKP2 expression scores were determined according to our previously published papers (9, 16).

Statistical analysis
The differences in results between groups were compared using ANOVA or Student’s t test. Data were expressed as mean ± SD. The correlation between PKC\textsubscript{i} and SKP2 expression levels was analyzed using the Spearman correlation test. All statistical analyses were performed with the SPSS 13.0 Statistical program for Windows. P values less than 0.05 were considered significant.

Results
RNAi of PKC\textsubscript{i} in EC9706 cells
EC9706 cells were used to explore the role of PKC\textsubscript{i} in ESCC in the current study because they are an ESCC cell line with PKC\textsubscript{i} overexpression (data not shown) and high metastatic ability. Two duplex PKC\textsubscript{i} siRNAs, targeting sequences in the 3’UTR and the coding region of the PKC\textsubscript{i} gene, respectively, PKC\textsubscript{i} siRNA-1 and siRNA-2, were chemically synthesized for transient downregulation of PKC\textsubscript{i} expression. After transient transfection for 48 hours,
PKCι siRNA-2 could both effectively suppress the expression of PKCι. Western blot analysis showed that PKCι siRNA-1 and siRNA-2 could both effectively suppress the expression of PKCι (Fig. 1B). Double-stranded oligonucleotides, synthesized according to PKCι siRNA-2, were inserted into PGCSI.6.neo.GFP shRNA expression vectors. As shown in Figure 3A, PKCι expression was suppressed in P4 and P8 clones. In this study, we refer to PKCι stably downregulation clones as PKCι RNAi. Parallel experiments were performed using the scramble sequence RNAi clones (S1 and S2). Stably transfected clones were used for in vitro assays and colony-formation assays.

**PKCι depletion decreased the motility of ESCC cells**

Our previous results showed that elevated expression of PKCι was correlated with lymph node metastasis in ESCC (9). Metastasis is a complicated procedure that involves many steps. Migration, invasion, and adhesion are all key components of metastasis, so we examined whether PKCι is involved in these stages. Haptotactic cell migration assays showed that after PKCι depletion, fewer cells migrated to the bottom of the chamber, compared with non-silencing and parental cells (Fig. 1A top and 1C). The invasive potential of the PKCι siRNA cells, as assessed by measuring the ability of cells to transverse a reconstituted basement membrane of Matrigel, was reduced when compared with that of the control cells (Fig. 1A, lower panel and 1C, bottom). Western blot analysis confirmed the suppression of PKCι expression in PKCι siRNA-transfected cells (Fig. 1B). These experiments were repeated 3 times with similar results.

Because the PKCι-induced increase in migration could be the result of an increase in the adhesion of tumor cells to the substrate, we evaluated the adhesive abilities of 3 groups of cells by measuring the number of cells attached to Matrigel. No significant differences were detected by MTS assays among the groups (Supplementary Fig. S1). Thus, knockdown of PKCι expression drastically suppressed the mobility of EC9706 cells in vitro.

**PKCι knockdown promoted cell anoikis via inactivation of the PI3K/AKT pathway**

We subsequently analyzed whether PKCι expression affected cell-cycle progression or apoptosis. Flow cytometric results showed that PKCι downregulation had no effect on these phenotypes (Supplementary Fig. S2 and S3). We investigated apoptosis of cells after detachment, because PKCι gene amplification and overexpression are associated with lymphoid metastasis (9) and anoikis is one type of apoptosis involved in tumor metastasis (25). The percentage of PKCι siRNA apoptotic cells was approximately 2-fold higher than the control and parental cells by Annexin V-FITC/propidium iodide staining (Fig. 2B, C). To confirm that the results of the siRNA experiments described before were due to the inhibition of PKCι expression, we expressed human PKCι as a transgene in PKCι siRNA cells. In this experiment, PKCι siRNA-1 was used to knock down PKCι expression because it targets the 3′UTR of endogenous human PKCι mRNA, which makes it possible to reconstitute PKCι expression in PKCι siRNA-1 cells using an

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**Figure 1.** Downregulation of PKCι expression decreased cell migration and invasion. A, representative photos of a haptotactic migration assay and a matrigel chemoinvasion assay in PKCι siRNA, nonsilencing siRNA, and parental EC9706 cells. B, Western blot analysis of PKCι expression in PKCι siRNA, nonsilencing siRNA, and parental cells after transient transfection. C, statistical plots of migration and matrigel chemoinvasion assay. The number of PKCι siRNA cells that transversed the Transwell membranes in the haptotactic migration assay and the matrigel chemoinvasion assay was significantly decreased, compared with nonsilencing siRNA and the parental cells. Columns, mean of 3 individual experiments; Bars, SD; *, P < 0.05.
exogenous human PKCι expression construct that lacks the 3'UTR targeting sequence. When a plasmid encoding the PKCι gene was transfected into PKCι siRNA-1 cells, reexpression of PKCι rescued the ability of PKCι siRNA-1 cells to inhibit anoikis (Fig. 2E, F, G), demonstrating a specific requirement for PKCι in the anoikis-resistance of EC9706 cells.

Molecular mechanisms of anoikis resistance have been described concerning several signaling pathways in different cell types. Our previous studies indicated that PI3K/AKT and MEK/Erk are 2 critical pathways mediating the anoikis of ESCC cells (16, 26, 27). In this study, we examined the potential effects of PKCι on the activation of PI3K/AKT and MEK/Erk pathways. After PKCι depletion and cell suspension, p-AKT expression was downregulated (Fig. 2D), and no significant differences in p-Erk expression were observed in the 3 groups. Furthermore, p-AKT expression was suppressed no matter whether PKCι-silenced cells

Figure 2. PKCι inhibition promoted anoikis via inactivation of the PI3K/AKT pathway. A, Western blot analysis of cell lysates of PKCι siRNA, nonsilencing siRNA, and parental cells for PKCι and β-actin after transient transfection. B, the percentage of apoptotic cells significantly increased in the PKCι siRNA group, compared with the nonsilencing siRNA and parental cells in an anoikis assay. Columns, mean of 3 individual experiments; Bars, SD; *, P < 0.05. C, flow cytometry data analysis of apoptotic cells by Annexin V-FITC/propidium iodide staining. After transient transfection, cells were cultured on polyHEMA-coated dishes for 24 hours. D, Western blot analysis of cell lysates of PKCι siRNA, nonsilencing siRNA, and parental cells for PKCι, p-AKT on Ser473, AKT, pErk on Tyr202, Erk, and β-actin after transient transfection and cell suspension for 24 hours. E, Western blot analysis of the expression level of PKCι in the nonsilencing, PKCι siRNA, and rescue groups. F, percentage of cells undergoing anoikis in the rescue group was significantly lower than that of the PKCι siRNA group. Columns, mean of 3 individual experiments; Bars, SD; *, P < 0.05. G, flow cytometry data analysis of apoptotic cells by Annexin V-FITC/propidium iodide staining. After transient transfection, cells were cultured on polyHEMA-coated dishes for 24 hours.
were attached, detached, or replated. In addition, forced transient PKCι expression in PKCι siRNA-1 cells could activate AKT phosphorylation in all 3 conditions, demonstrating a specific requirement for PKCι in AKT activation (Fig. 5C, D, E).

**Knockdown of PKCι inhibited colony formation of ESCC cells in soft agar and s.c. tumor growth in nude mice**

To investigate the effect of PKCι expression on tumor growth, we examined in vitro cell growth rate using MTS cell proliferation assays. No significant changes in growth rate were observed between PKCι RNAi and scramble sequence RNAi cells (Fig. 3B). By contrast, depletion of PKCι significantly blocked the ability of EC9706 cells to grow as anchorage-independent colonies in soft agar (Fig. 3C, D). Tumor formation assays in nude mice were performed to investigate the effect of PKCι expression on tumor growth in vivo. PKCι RNAi and scramble sequence RNAi cells were inoculated into 6 nude mice in each group. Both groups developed tumors, but the tumors formed from the scramble sequence RNAi cells grew faster than did.
the PKCᵢ RNAi tumors. The mice were sacrificed 4 weeks after injection. Tumors formed from scramble sequence RNAi cells were significantly heavier than tumors formed from PKCᵢ RNAi cells (P < 0.05; Fig. 3E, F). Immunohistochemical staining confirmed the presence of PKCᵢ expression in the formalin-fixed, paraffin-embedded sections of the s.c. tumors (Fig. 3G).

**PKCᵢ inhibition decreased lung metastases in nude mice**

Given that increased cell migration and suppression of anoikis both contribute to the metastatic potential of cancer cells, we tested whether inhibition of PKCᵢ would affect the ability of EC9706 cells to metastasize. PKCᵢ RNAi and scramble sequence RNAi cells were introduced via tail vein into 8 nude mice from each group. Eleven weeks after injection, the animals were sacrificed and examined for the presence of lung metastases. In the scramble sequence RNAi group, 2 mice developed visually observable lung nodules and another 2 mice developed small tumor nodules that could be observed only under the microscope in H&E sections of the lungs. However, no tumor nodules were found either visibly or under the microscope in H&E sections of mouse lungs in the PKCᵢ RNAi group. Representative lungs and their corresponding H&E staining are shown in Figure 4A. The average number of lung metastases of the mice injected with PKCᵢ RNAi cells was significantly lower than that of mice injected with scramble sequence RNAi cells (P < 0.05; Fig. 4B). These results suggest that PKCᵢ expression promotes tumor metastases in vivo.

**PKCᵢ regulated resistance to anoikis through SKP2**

Our previous study suggested that SKP2 was also implicated in PI3K/AKT-mediated anoikis resistance in EC9706 cells (16). Therefore, we hypothesized that there may be a regulating relationship between SKP2 and PKCᵢ. Western blot analysis in this study indicated that SKP2 expression did not change in adherent cells after PKCᵢ knockdown (Fig. 5A), but was markedly decreased in detached cells (Fig. 5B). To verify that PKCᵢ directly controlled SKP2 expression, we transfected vectors encoding the PKCᵢ gene into PKCᵢ siRNA-1 cells, and transfected cells were placed in attached, detached, and replated states. Expression of exogenous PKCᵢ restored intracellular level of the SKP2 protein only in the detached cells (Fig. 5C, D, E), which confirmed that PKCᵢ could only regulate SKP2 expression in detached ESCC cells. When plasmids encoding the SKP2 gene were transfected into PKCᵢ siRNA-1 cells, elevated SKP2 expression rescued the ability of PKCᵢ siRNA-1 cells to inhibit anoikis. These data revealed a novel required role for PKCᵢ-SKP2 signaling in ESCC cells anoikis-resistance (Fig. 5F, G).

**PKCᵢ regulated SKP2 protein expression through the ubiquitin-proteasome pathway**

RT-PCR results showed that SKP2 mRNA level was not changed in detached cells with decreased PKCᵢ. However, the addition of the proteasome inhibitor MG132 could prevent the decreased SKP2 protein level in detached cells after PKCᵢ depletion (Fig. 6A). To further support these results, immunoprecipitation experiments with anti-SKP2 antibody were utilized, followed by immunoblotting with anti-ubiquitin antibody. In the presence of MG132, the ubiquitin was observed in the SKP2 immunocomplex, and its amount was substantially increased in the PKCᵢ knockdown group (Fig. 6B, top). Western blot analysis (Fig. 6B, bottom) confirmed PKCᵢ knockdown and ubiquitination assay loading control.

The aforementioned results indicate that PKCᵢ activated AKT phosphorylation and enhanced the intracellular level of SKP2 protein in detached ESCC cells. In addition, recent reports have suggested that the PI3K/AKT pathway regulates SKP2 expression level by one or more unknown mechanisms (28–30). Based on these data, we hypothesized that PKCᵢ might regulate SKP2 expression through AKT signaling. To investigate this, we treated EC9706 cells with the PI3K inhibitor LY294002. As shown in Figure 6C and D, the loss of p-AKT revealed robust inhibition of PI3K.
activity. However, inhibition of PI3K activity did not result in decreased of SKP2 protein expression either in attached or detached cells, demonstrating that the PI3K/AKT pathway did not regulate SKP2 expression in our experiment.

Interestingly, we found decreased PKCι protein level concomitant with the loss of phosphorylation by AKT.

**Correlation between PKCι and SKP2 expression in ESCC tissues**

To determine the relationship between PKCι and SKP2 in tumor tissues, we analyzed their protein expression using an immunohistochemical approach combined with tissue microarray. Immunohistochemistry results showed that of...
133 specimens tested, all normal epithelia had negative or weak PKCι and SKP2 expression, whereas 50 (37.6%) of the tumors had more intense staining of PKCι, and 40 (30.1%) exhibited high SKP2 expression than normal tissues. PKCι expression was significantly correlated with SKP2 expression in the tumors ($P = 0.031$, Table 1 and Fig. 7).

**Discussion**

We previously reported that both frequent amplification and overexpression of PKCι were associated with lymph node metastasis in ESCC, suggesting that PKCι might play a key role in the progression of the disease (9). To evaluate this possibility, we first assessed PKCι protein expression in
a panel of ESCC cell lines. We chose EC9706 cells for the PKC\textit{i} functional studies because they were shown to have high PKC\textit{i} expression (data not shown) and are frequently used in ESCC metastasis research (16, 26, 27).

Previous studies suggested that PKC\textit{i} was involved in nicotine-activated migration and invasion of H1299 human non–small-cell lung cancer cells and promoted glioblastoma cell motility and invasion by repression of RhoB expression (31, 32). To explore the effect of PKC\textit{i} on ESCC cell migration and invasion, we performed transient transfections with 2 PKC\textit{i}-targeted siRNAs in EC9706 cells. Both PKC\textit{i} siRNAs significantly suppressed PKC\textit{i} expression and induced both inhibition of cell migration and invasion of ESCC cells.

Anoikis, one type of apoptotic cell death due to the loss of cell adherence, is critical for the regulation of tissue homeostasis in tissue remodeling, development, fibrosis, and tumor metastasis (33). Here, we showed that decreased PKC\textit{i} expression was associated with an increase in anoikis in ESCC cells and that reexpression of PKC\textit{i} could reestablish anoikis resistance in PKC\textit{i}-silenced cells. We postulate that PKC\textit{i} overexpression might help malignant cells survive in an anchorage-independent manner, leading to metastasis in ESCC patients. Molecular mechanisms of anoikis resistance have been described concerning several signaling pathways in different cell types. Our previous studies indicated that PI3K/\textit{akt} and MEK/Erk are 2 critical pathways mediating the anoikis of ESCC cells (16, 26, 27). We revealed that after PKC\textit{i} inhibition, AKT phosphorylation was decreased, whereas no difference in Erk activation was observed. Moreover, reexpression of PKC\textit{i} could activate AKT phosphorylation. These data indicated that PI3K/\textit{akt} signaling was the main downstream target responsible for the protective effect of PKC\textit{i} against anoikis in ESCC cells. Interestingly, our recent investigation revealed that SKP2 was also implicated in PI3K/\textit{akt}-mediated anoikis resistance in ESCC cells (16). We therefore tested the hypothesis that SKP2 may participate in PKC\textit{i}-associated antianoikis signaling pathways. Our data showed that, in detached ESCC cells, PKC\textit{i} repression lowered SKP2 expression, whereas reexpression of PKC\textit{i} in PKC\textit{i}-silenced cells could enhance intracellular level of SKP2 protein. In addition, SKP2 knockdown did not affect PKC\textit{i} protein expression (data not shown), indicating that PKC\textit{i} acts upstream of SKP2. Moreover, elevation of SKP2 can reestablish cellular resistance to anoikis in ESCC cells with PKC\textit{i} knockdown. Taken together, these results suggest that in detached ESCC cells, PKC\textit{i} overexpression elevates the expression of SKP2 protein, promotes activation of the PI3K/\textit{akt} pathway, and increases resistance to anoikis in ESCC. PKC\textit{i} expression was significantly correlated with SKP2 in 133 ESCCs ($P = 0.031$), which confirmed the regulation between PKC\textit{i} and SKP2 \textit{in vivo}. To our knowledge, this is the first report that PKC\textit{i} is involved in cell anoikis through SKP2 regulation, which extends our understanding of the roles of PKC\textit{i} and SKP2 in tumor metastasis.

To explore the possible mechanisms of PKC\textit{i} regulation of SKP2 expression, we first determined the SKP2 mRNA level in suspended PKC\textit{i}-siRNA cells by RT-PCR and found that the SKP2 mRNA expression was unchanged after PKC\textit{i} knockdown. Previous studies had suggested that SKP2 transcription was regulated by several important signaling pathways (29, 34). However, our data showed that PKC\textit{i} did not regulate SKP2 at the transcriptional level in ESCC cells. We then postulated that PKC\textit{i} might regulate SKP2 expression on the posttranscriptional level. When suspended cells were treated with the proteasome inhibitor MG132, the decrease in SKP2 protein expression after PKC\textit{i} depletion disappeared, which strongly suggested that PKC\textit{i} might regulate SKP2 expression via proteasomes. To confirm this hypothesis, an \textit{in vivo} ubiquitination assay was performed. The results indicated that SKP2 degradation initiated by PKC\textit{i} depletion was mediated via the ubiquitin-proteasome pathway.

Cdh1 has been revealed as an E3 ligase that promotes SKP2 destruction (17, 18). Several studies have reported on the relationship between the PI3K/\textit{akt} pathway and SKP2 (28–30). It has been observed that in 293T cells AKT1 phosphorylates SKP2 at Ser 72, which is required to disrupt the interaction between Cdh1 and SKP2, and as a result prevents SKP2 degradation by the APC-Cdh1 ubiquitin ligase complex (35, 36). In the present study, we found that PKC\textit{i} could activate AKT phosphorylation. Based on previous reports and our data, we hypothesized that PKC\textit{i} might regulate SKP2 expression through AKT signaling. However, the addition of LY294002 did not change SKP2 protein level either in attached or detached EC9706 cells, indicating that the PI3K/\textit{akt} pathway did not regulate

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<th>PKC\textit{i}</th>
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</table>

NOTE: Correlation coefficient $= 0.202$, $P = 0.031$
SKP2 expression in our experiments. In addition, coimmuno precipitation analysis showed that AKT1 did not interact with SKP2 (Supplementary Fig. S4B). Our previous study found that decreased SKP2 reduced p-AKT expression and that the PI3K/AKT pathway is the downstream target of SKP2 (16). Our previous and current data showed that SKP2 acts upstream of AKT, and PKC\(\iota\) might regulate SKP2 protein degradation through other molecules but not PI3K/AKT pathway. Moreover, Mao and colleagues cotransfected breast cancer BT-549 cells with expression constructs encoding V5-tagged PKC\(\iota\)/i and HA-tagged AKT and then assessed coimmunoprecipitation of the transfected PKC\(\iota\)/i with AKT. They observed that exogenous PKC\(\iota\)/i was present at low level in AKT immunoprecipitates, indicating that PKC\(\iota\)/i interacts with AKT in breast cancer cells (37). We did not observe PKC\(\iota\), AKT1 and SKP2 coprecipitation in detached EC9706 cells in the present study (Supplementary Fig. S4A). These differential observations in our and other experiments might reflect that there exist distinct signaling networks in different cell lines. The detailed mechanism underlying PKC\(\iota\) regulation of SKP2 through the ubiquitin-proteasome pathway remains to be elucidated. Interestingly, we found that the addition of LY294002 also resulted in a decrease in PKC\(\iota\) protein level, indicating a possible negative feedback loop between PI3K/AKT and PKC\(\iota\) that may help to maintain the balance between cell survival and apoptosis.

In conclusion, our results provide evidence that PKC\(\iota\) promotes tumor metastasis through increased cell migration ability and anoikis resistance in ESCC. PKC\(\iota\) enhances resistance to anoikis via the PKC\(\iota\)-SKP2-AKT pathway. The data suggest that PKC\(\iota\) is a candidate oncogene involved in the progression of ESCC and that SKP2 is a downstream effector of PKC\(\iota\) signaling in ESCC.
Disclosure of Potential Conflicts of Interest

No potential conflicts of interest were disclosed.

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Atypical Protein Kinase C{\textsubscript{t}} (PKC{\textsubscript{t}}) Promotes Metastasis of Esophageal Squamous Cell Carcinoma by Enhancing Resistance to Anoikis via PKC{\textsubscript{t}}-SKP2-AKT Pathway

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