Effects of Raf Kinase Inhibitor Protein Expression on Metastasis and Progression of Human Epithelial Ovarian Cancer

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Abstract

Loss of function of metastasis suppressor genes is an important step in the progression to a malignant tumor type. Studies in cell culture and animal models have suggested a role of Raf kinase inhibitor protein (RKIP) in suppressing the metastatic spread of prostate cancer, breast cancer, and melanoma cells. However, the function of RKIP in ovarian cancer (OVCA) has not been reported. To explore the potential role of RKIP in epithelial OVCA metastasis, we detected the expression levels of RKIP protein in tissue samples from patients with epithelial OVCA. Consequently, the expression of RKIP is reduced in the poorly differentiated OVCA than in the well-differentiated and moderately differentiated OVCA. In addition, in vitro cell invasion assay indicated that the RKIP expression was inversely associated with the invasiveness of five OVCA cell lines. Consistent with this result, the cell proliferation, anchorage-independent growth, cell adhesion, and invasion were decreased in RKIP overexpressed cells but increased in RKIP down-regulated cells. Further investigation indicated that RKIP inhibited OVCA cell proliferation by altering cell cycle progression rather than promoting apoptosis. Furthermore, the overexpression of RKIP suppressed the ability of human OVCA cells to metastasize when the tumor cells were transplanted into nude mice. Our data show the effect of RKIP on the proliferation, migration, or adhesion of OVCA cells. These results indicate that RKIP is also a metastasis suppressor gene of human epithelial OVCA.


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Introduction

Metastasis is defined as the formation of progressively growing secondary tumor foci at sites discontinuous from the primary lesion (1). The metastatic process involves a complex cascade of events. In brief, a metastatic cancer cell must escape from the primary tumor, enter the circulation, arrest in the microcirculation, invade a different tissue compartment, and then grow at that secondary site. Theoretically, metastasis could be blocked by inhibiting a single gene, called metastasis suppressor gene, which allows the completion of any of those steps in the metastatic cascade (1–5). Several metastasis suppressor genes (e.g., Nm23, KISS1, KAI1, BRMS1, TIMPs, E-cadherin, MMK4, TXNIP, CRSP3, DRG-1, and RhoGD12) have been identified (6) and recent studies showed that the Raf-1 kinase inhibitor protein (RKIP) is a novel metastasis suppressor gene. RKIP levels were found to be reduced or absent in variants of established cell lines derived from metastatic prostate cancer (7), breast cancer (8, 9), and melanoma cells (10, 11). Reconstitution of RKIP expression prevented the invasion into the Matrigel (7, 10) and the metastasis in an orthotopic prostate cancer mouse model (7) but not the growth of the primary tumors. Moreover, RKIP restored expression sensitized human prostate and breast cancer cell lines to chemotherapy-triggered apoptosis (8).

RKIP was first identified as an interacting partner of Raf-1 and as a negative regulator of the mitogen-activated protein kinase (MAPK) cascade initiated by Raf-1 (12). This pathway is involved in the regulation of many fundamental cellular processes, including proliferation, differentiation, survival, and cell death. The key players are three kinases: Raf, which phosphorylates and activates MAPK/ extracellular signal-regulated kinase (ERK) kinase (MEK), which phosphorylates and activates ERK (13, 14). RKIP interferes with Raf-1–mediated phosphorylation and the activation of MEK by disrupting the interaction between the two kinases (15).

The molecular changes associated with acquisition of metastatic ability in ovarian cancer (OVCA) are poorly understood. Both OVCA and prostate cancer are endocrine-related and hormone-dependent tumors. There is a cross-talk between endocrine, signal transduction, and regulatory pathways in normal and neoplastic ovarian and prostatic tissues (16). Although the metastatic patterns of ovarian and prostate cancer are clinically different, we speculated that the fundamental mechanisms regulating the metastatic colonization would be conserved. More important is the observation that loss of 12q is a frequent event in human epithelial OVCA (17–19), whereas
RKIP is located on human chromosome 12q24.22. Therefore, we hypothesize that RKIP would play an important role in the metastasis of human epithelial OVCA. To address whether RKIP is related to the metastatic behavior in human epithelial OVCA, we investigated RKIP expression in different ovarian epithelial tumors, which indicated an inverse correlation tendency between RKIP protein expression and the malignant degree of ovarian epithelial tumors. Reconstitution of RKIP levels in OVCA cells by exogenous expression impaired in vitro invasiveness, whereas down-regulation of RKIP expression by antisense DNA promoted invasiveness. Thus, RKIP seems to be a potential metastasis suppressor gene of human epithelial OVCA.

**Results**

**RKIP Expression in Human Ovarian Epithelial Tumors**

To examine RKIP expression and distribution in vivo, immunohistochemical approach was done to detect the RKIP in clinical samples of human ovarian epithelial tumors, including 17 samples of benign ovarian epithelial tumor and 79 samples of epithelial OVCA. RKIP was detectable in almost all benign ovarian epithelial tumors with only one exception (n = 16 of 17). Intense RKIP staining was also observed in well-differentiated (n = 22 of 38) and moderately differentiated (n = 15 of 26) epithelial OVCA but was rare in poorly differentiated samples (n = 2 of 15). RKIP protein was found to be predominantly cytoplasmic distribution. The intensity of RKIP-positive cells was reduced in epithelial OVCA compared with benign ovarian epithelial tumor. In poorly differentiated OVCA, an additional reduction of expression was seen with the weak staining or no RKIP staining at all (P < 0.001, Fisher’s exact test) compared with well-differentiated or moderately differentiated OVCA (Table 1). Representative sections are presented in Fig. 1. These data show an inverse correlation between RKIP protein expression and malignant degree of ovarian epithelial tumors, as the intensity of RKIP protein in poorly differentiated OVCA is significantly reduced than in well-differentiated or moderately differentiated OVCA.

**Table 1. Expression of RKIP in Human Ovarian Epithelial Tumors**

<table>
<thead>
<tr>
<th>Tissue</th>
<th>RKIP Expression</th>
<th>Total, n (%)</th>
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<tbody>
<tr>
<td></td>
<td>Negative, n (%)</td>
<td>Weak, n (%)</td>
</tr>
<tr>
<td>Benign ovarian epithelial tumor*</td>
<td>1 (5.9)</td>
<td>4 (23.5)</td>
</tr>
<tr>
<td>Epithelial OVCA</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Well differentiated</td>
<td>5 (13)</td>
<td>11 (30)</td>
</tr>
<tr>
<td>Moderately differentiated †</td>
<td>2 (7.7)</td>
<td>9 (35)</td>
</tr>
<tr>
<td>Poorly differentiated †</td>
<td>11 (73.4)</td>
<td>2 (13.3)</td>
</tr>
</tbody>
</table>

NOTE: The staining intensity was scored based on the amount of stain detected as follows: negative, 0; weak, 1; moderate, 2; or strong, 3. *P = 0.913, compared with well-differentiated OVCA; P = 0.656, compared with moderately differentiated OVCA; P = 0.009, compared with poorly differentiated OVCA (Fisher’s exact test).
† P = 0.823, compared with well-differentiated OVCA (Fisher’s exact test).
‡ P = 0.000, compared with well-differentiated OVCA; P = 0.001, compared with moderately differentiated OVCA (Fisher’s exact test).

**FIGURE 1.** Immunohistochemical staining for RKIP in human ovarian epithelial tumors. Paraffin-embedded sections of human ovarian epithelial tumors were immunostained with anti-RKIP antibody (brown) and counterstained with hematoxylin (blue) and photographed at ×100 or ×200 magnification. A. Black arrows, strong RKIP staining in benign ovarian epithelial tumor. Magnification, ×100. B. Blowup of part of the section in A. Magnification, ×200. C. Moderate RKIP staining in epithelial OVCA (well differentiated). Magnification, ×100. D. Blowup of part of the section in C. Magnification, ×200. E. Negative or weak RKIP staining in epithelial OVCA (poorly differentiated). Magnification, ×100. F. Blowup of part of the section in E. Magnification, ×200.
In vitro Invasiveness of RKIP in OVCA Cell Lines

To investigate the potential role of RKIP in OVCA, we first detected the expression panel of RKIP in five human OVCA cell lines. As shown in Fig. 2, the mRNA level of RKIP was basically consistent with protein expression level in these five cells. Compared with HO-8910PM cells (Fig. 2A), the RKIP mRNA level was variably increased in OVCAR-3, CAOV-3, SKOV-3, and HO-8910 cells (3.8-, 3-, 1.8-, and 1.3-fold, respectively). Accordingly, the protein level of RKIP was also higher in OVCAR-3, CAOV-3, SKOV-3, and HO-8910 cells (3.1-, 2.1-, 1.8-, and 1.5-fold, respectively) compared with HO-8910PM cells (Fig. 2B). To further investigate the expression and distribution of RKIP at cellular level, high-metastatic human OVCA cell line HO-8910PM and the parental low-metastatic cell line HO-8910 were immunostained with RKIP antibody. As shown in Fig. 2, RKIP protein was mainly expressed in the cytoplasm and close to the nucleus, and that the amount of RKIP-positive cells was lower in high-metastatic HO-8910PM cells (24.85 ± 1.59%; Fig. 2C) than in the parental low-metastatic HO-8910 cells (42.05 ± 2.77%; Fig. 2D; \( P < 0.05 \), Pearson’s \( \chi^2 \) test).

To investigate the association of metastatic phenotype and the expression level of RKIP, we also examined the \( \textit{in vitro} \) invasiveness of five OVCA cells by doing \( \textit{in vitro} \) cell invasion assay. As shown in Fig. 2E, different invasiveness was observed in HO-8910 and SKOV-3 cells as well as in HO-8910PM, CAOV-3, and OVCAR-3 cells, respectively (\( P < 0.05 \), one-way ANOVA). It suggested that RKIP expression was inversely associated with the invasiveness of five OVCA cells \( \textit{in vitro} \). The inverse correlation tendency between RKIP expression in OVCA cells and their \( \textit{in vitro} \) invasive ability indicates that RKIP is likely to be a metastasis suppressor gene in OVCA.

The Role of RKIP Expression in the Modulation of Raf/MEK/ERK Signaling in OVCA Cells

To further explore the role of RKIP in OVCA cells, the human OVCA cell line SKOV-3 was used to reconstitute the expression of RKIP by stable overexpression (Ss-RKIP) or down-regulation (As-RKIP) of RKIP. Meanwhile, SKOV-3 cells were transfected with pcDNA3.1(+) or pcDNA3.1(−) vector alone to be a negative control. The RKIP protein levels in these stable cells were determined by Western blot analysis. As shown in Fig. 3A, the RKIP protein levels in Ss-RKIP

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SKOV-3 cells were significantly increased compared with pcDNA3.1(+) or pcDNA3.1(-) control SKOV-3 cells. In contrast, RKIP protein levels were down-regulated in As-RKIP SKOV-3.

It has been reported that RKIP is able to inhibit the Raf-1/MEK/ERK pathway. To investigate the effect of RKIP on the activation steps of the Raf/MEK/ERK cascade in OVCA, we examined the protein levels and phosphorylation state of MEK and ERK. As shown in Fig. 3B, Ss-RKIP reduced basal phosphorylation of both MEK and ERK, whereas As-RKIP enhanced phosphorylation of both MEK and ERK compared with controls. These data indicate that RKIP is able to block basal levels of MEK and ERK activation in OVCA cells.

**FIGURE 3.** A. Expression levels of RKIP in different SKOV-3 clones of stably transfected cells, including untransfected SKOV-3 cells, SKOV-3 cells stably overexpressing RKIP protein (Ss-RKIP SKOV-3), SKOV-3 cells down-regulated expression of RKIP (As-RKIP SKOV-3) or with empty vector as control (control SKOV-3). Total cell lysates (40 μg) were loaded in each lane. β-Actin was used as an internal control for loading. Band intensity was quantified using densitometry and normalized to β-actin band intensity. B. RKIP affects MAPK activity in OVCA cells. The phosphorylation status of MEK and ERK in different SKOV-3 cells was detected by Western blot. Membranes were first probed with antibodies specific for the phosphorylated forms of MEK (p-MEK) or ERK (p-ERK) and then stripped and reprobed with antibodies against MEK or ERK, respectively, to detect the total MEK or ERK. Results are reported as band intensity relative to untransfected SKOV-3 cells, which were set at an expression level of 1. The experiments are representative of three independent experiments with similar results. C. RKIP inhibits OVCA cell proliferation in vitro. SKOV-3 cells and their transfectants were cultured in 96-well plates at 2 x 10^4 per well for 24, 48, 72, 96, and 120 h. The cell growth was assessed by MTT assay as described above. Points, mean of two separate experiments with sextuple samples; bars, SD.

**Effect of RKIP Expression on Biological Behaviors in OVCA Cells**

To examine whether modulation of RKIP expression influenced the tumorigenic properties of the OVCA cells, we measured the abilities of *in vitro* cell proliferation and anchorage-independent growth in Ss-RKIP SKOV-3, As-RKIP SKOV-3, or control SKOV-3 by 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assay and soft agar colony formation assay. The control SKOV-3, parental SKOV-3, as well as Ss-RKIP and As-RKIP SKOV-3 cells were grown in culture for 5 days. As shown in Fig. 3C, the ability of cell proliferation in the Ss-RKIP SKOV-3 cells was decreased compared with control SKOV-3 or parental SKOV-3 but increased in As-RKIP SKOV-3 cells. The effect of RKIP on OVCA cell proliferation was confirmed by fluorescence-activated cell sorting analysis of synchronized SKOV-3 cells, which revealed that Ss-RKIP cells had a significant increase in the G1 phase and decrease in the G2 + S phase compared with the control SKOV-3 or parental SKOV-3, whereas As-RKIP SKOV-3 cells had a significant decrease in the G1 phase and increase in the G2 + S phase (Fig. 4). On the other hand, the apoptotic peaks were not significantly altered by the ectopic expression of RKIP.
expression or down-regulation of RKIP (Fig. 4A-H). Thus, these data suggest that RKIP inhibits OVCA cell proliferation by altering cell cycle distribution rather than promoting apoptosis.

The soft agar colony formation assay further confirmed that RKIP inhibited OVCA cell proliferation. As shown in Fig. 5A to I, a significant decrease in the number of colonies formed by Ss-RKIP SKOV-3 cells compared with control SKOV-3 or parental SKOV-3, whereas a significant increase in the number of colonies formed by As-RKIP SKOV-3 cells. These data indicate that RKIP expression inhibits anchorage-independent growth of OVCA cells in soft agar.

Adhesion and invasion are two of the key components of the metastatic cascade. Accordingly, to examine whether RKIP expression is associated with adhesion and invasiveness of OVCA, in vitro adhesion assay and in vitro invasion assay were done to evaluate the adhesive and invasive abilities of the parental SKOV-3 cells and Ss-RKIP and As-RKIP SKOV-3 cells. As shown in Fig. 5J, the ability of cell adhesion in the Ss-RKIP SKOV-3 cells was decreased compared with control SKOV-3 or parental SKOV-3 (P < 0.001) but increased in the As-RKIP SKOV-3 cells (P < 0.001). Overexpression of RKIP led to an average of 37.3% decreased invasiveness in different Ss-RKIP SKOV-3 clones (Fig. 6C and D) compared with different control SKOV-3 clones [pcDNA3.1(+)] and pcDNA3.1(−); Fig. 6A and B] or parental SKOV-3 (Fig. 6G). Conversely, down-regulated RKIP expression caused an average of 163.2% increase in in vitro invasive ability in different As-RKIP SKOV-3 clones (Fig. 6E and F). These results suggest that RKIP expression is inversely associated with the adhesion and the invasiveness of OVCA cells in vitro.

Ectopic RKIP Expression Suppresses Metastatic Ability of SKOV-3 Cells

To examine the effect of RKIP-mediated metastasis suppression in vivo, the Ss-RKIP SKOV-3 cells that stably

FIGURE 4. Effect of RKIP on cell cycle distribution of OVCA cells. Cell cycle distribution was detected by flow cytometric analysis with propidium iodide staining. A to H. The flow cytometric analysis is a representative of three independent experiments with similar results. I. Results were expressed as the percentage of cells in G1 phase or G2 + S phase and are from three separate experiments. #, P > 0.05; **, P < 0.001, compared with the parental cells.
overexpress RKIP, or SKOV-3 cells transfected with pcDNA3.1(+) as control, were injected intraperitoneally into nude mice (n = 5 per clone), respectively. Twenty days after injection, the mice were sacrificed to evaluate the effect of ectopic RKIP expression on metastatic colonization. Necropsies revealed widespread dissemination of tumor cells in animals that received injections of the control SKOV-3 cells, including multiple and large adherent and nonadherent metastatic nodules throughout the peritoneum, stomach, duodenum, liver, spleen, and diaphragm (Fig. 7A-E). Conversely, SKOV-3ip.Ss-RKIP group developed less and small solitary tumor masses in the peritoneal cavity (Fig. 7F-J). The statistic analysis showed significant difference in the mean number (Table 2) or the mean size (Table 3) of the tumors of these two groups of mice. As shown in Table 2, the mean number of the tumor masses in mice injected with control SKOV-3 cells was 66.8 ± 8.438 compared with the mean number in mice injected with the Ss-RKIP SKOV-3 cells, which was 26.00 ± 4.95. The mean size of the tumor masses in mice injected with control SKOV-3 cells was 9.297 ± 2.967 mm³ compared with the mean size in mice injected with the Ss-RKIP SKOV-3 cells, which was 3.493 ± 2.927 mm³ (Table 3). These data indicate that ectopic expression of RKIP in the SKOV-3 cells decreases the metastatic ability, which is consistent with our present results that the expression of the RKIP is inversely correlated with the metastatic potential of some established OVCA cell lines. In addition, the expression of RKIP is reduced in the poorly differentiated OVCA than in the well-differentiated and moderately differentiated OVCA.

Discussion

OVCA is a common cause of cancer death. The major reason for the mortality of this cancer is the high metastatic ability. However, the mechanism through which the primary OVCA cells colonize to remote sites is not clear. A growing number of genes have been identified to be metastasis-promoting genes,

FIGURE 5. A to I. RKIP suppresses anchorage-independent growth of OVCA cells in soft agar. SKOV-3 cells and their transfectants were assayed for their ability to proliferate in growth medium containing 0.3% agar and the formation of multicellular colonies photographed at x40 magnification after 14 d from six-well plates in triplicate. Data are representative of three independent experiments. Columns, mean colony numbers per well; bars, SD. *, P < 0.05; **, P < 0.01, compared with the empty vector pcDNA3.1(+) -transfected cells; #, P < 0.05; ##, P < 0.01, compared with the empty vector pcDNA3.1(−)-transfected cells. J. RKIP suppresses in vitro cell adhesion of OVCA cells. The in vitro adhesion of SKOV-3 cells and their transfectants was measured by determined cell counts that adhered to Matrigel-coated 96-well plates after washing with PBS by MTT assay as described above. Columns, mean of three separate experiments with sextuple samples; bars, SD. #, P > 0.05; **, P < 0.001, compared with the parental cells.
including VEGF, MMPs, Ras, Mts1, and Mta1. In contrast, the metastasis suppressor gene may inhibit early steps in metastatic colonization. Thus, identification of metastasis suppressor gene would provide the advantage to prevent the metastasis.

Recently, RKIP was identified as a metastasis suppressor gene, which was lowly expressed or lost in human prostate cancer (7, 20), breast cancer (8, 9), melanoma cells (10, 11), and insulinomas (21). However, the expression of RKIP in OVCA has not been previously reported. In this contribution, we first show that the majority of the poorly differentiated OVCA specimens show significantly decreased expression of RKIP compared with well-differentiated, moderately differentiated, or benign OVCA specimens, suggesting that RKIP expression levels are down-regulated in poorly differentiated OVCA cells, which have high risk of metastasis. Furthermore, the observation that the inverse correlation between RKIP protein expression and malignant degree of ovarian epithelial tumors suggests that RKIP possibly acts as a useful marker for estimating malignant degree of ovarian epithelial tumors. However, the etiology of down-regulation or loss of RKIP expression in OVCA is unknown. Chatterjee et al. (8) reported that deletion, mutation, or promoter hypermethylation of the RKIP gene was not responsible for down-regulation of RKIP in melanoma cell lines. Thus, further studies need to be done to determine if RKIP down-regulation or loss is due to deletion/mutation of the RKIP gene or due to epigenetic mechanisms that regulate RNA or protein levels of RKIP.

Metastasis is a highly organized process that consists of induction of cell motility, invasion to secondary site, and adhesion to the extracellular matrix. The invasive ability is an essential property of metastasis suppressor gene, which is prerequisite for tumor metastasis. To determine the role of RKIP in the metastasis of epithelial OVCA, we first investigated the association between RKIP expression and the in vitro invasiveness in five OVCA cell lines. We found that RKIP expression was inversely associated with the invasiveness of five OVCA cells in vitro. In addition, the expression level of RKIP was lower in high-metastatic HO-8910PM cells than in their parental low-metastatic HO-8910 cells. These findings indicate that RKIP is likely to be a metastasis suppressor gene in OVCA.

SKOV-3 cells were established from ascitic fluid collected from patient with OVCA and could form overt metastases in nude mice (22). In addition, we found that the expression level of RKIP was relatively low, whereas the in vitro invasiveness was relatively high. Regardless of the exact mechanism of RKIP in OVCA metastatic process, RKIP cDNA or antisense DNA was transfected into SKOV-3 cells to reconstitute the expression of RKIP, and then focusing on the changes in the characteristics of cell proliferation, adhesion, and invasiveness, all of which are essential steps for the establishment of metastasis. In RKIP overexpressed cells (Ss-RKIP SKOV-3), the cell proliferation, anchorage-independent growth, cell adhesion, and cell invasion were decreased compared with control cells (control SKOV-3), whereas the values for the same assays were increased in RKIP down-regulated cells (As-RKIP SKOV-3). Adhesion is considered to have a key role in regulating cell growth at the metastatic secondary site. In this

![FIGURE 6. RKIP inhibits in vitro invasiveness of OVCA cells. The in vitro invasion of SKOV-3 cells and their transfectants was measured by determined cell counts that penetrated through Matrigel-coated Transwell chambers (8-μm pore size) and photographed at ×200 magnification. #, P > 0.05; **, P < 0.001, compared with the parental SKOV-3 cells.](image-url)
study, we first show that RKIP expression affects adhesive ability of OVCA cells by in vitro cell adhesion assay. Thus, upregulated expression of RKIP is likely to inhibit the growth of the cancer cells in the metastatic sites due to a decrease in cell adhesive ability.

It is the first report that RKIP expression suppresses cell proliferation and anchorage-independent growth of OVCA, as evidenced by MTT assay, fluorescence-activated cell sorting analysis, and soft agar colony formation assay. In addition, we found that RKIP inhibited OVCA cell proliferation by altering cell cycle progression rather than by promoting apoptosis. This finding is in accordance with the study of Zhang et al. (21) in insulinomas but is different from the studies of Fu et al. (7) and Schuierer et al. (10), who reported, respectively, that RKIP had no effect on cell proliferation of prostate cancer and melanoma. The suppressive effect of RKIP on anchorage-independent growth of OVCA cells is in accordance with the results of Park et al. (11) in melanoma cells but is different from the results of Fu et al. (7), who reported that RKIP had no effect on anchorage-independent growth of prostate cancer cells.

Table 2. Metastatic Ability of SKOV-3ip.Control and SKOV-3ip.Ss-RKIP

<table>
<thead>
<tr>
<th>Organ</th>
<th>Cell Lines</th>
<th>Mean Number of Metastases on Day 20 ± SD</th>
<th>P</th>
</tr>
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<tbody>
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<td>Peritoneum</td>
<td>Ss-RKIP-SKOV-3</td>
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<tr>
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<td>Control</td>
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<tr>
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<td>Control</td>
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FIGURE 7. Ectopic RKIP expression suppresses metastatic ability in vivo. Metastases in female nude mice injected intraperitoneally with 5 × 10⁶ control SKOV-3 cells [pcDNA3.1(+) vector control] or Ss-RKIP SKOV-3 cells (ectopic RKIP expression). The mice were sacrificed 20 d after injection. Photographs A to E represent control group. Photographs F to J represent ectopic RKIP expression group. A. White arrow, large amount of tumor mass implants is observed near the liver and the stomach area. Box, numerous metastases are present along the small bowel surface. B. Inset of A. White arrow, magnified view of small bowel implants. C. Yellow arrow, magnified view of diaphragm implants. D. Inset of A. Yellow arrow, magnified view of the implants near the liver and stomach. E. Arrowhead, implants on the epiploon. F to J show fewer and small amount of tumor implants in small bowel area (F and G), diaphragm (H), liver (I), and epiploon (J).
suppressive effect of RKIP on invasiveness of OVCA cells is in accordance with the results of Fu et al. (7) in prostate cancer cells and Schuierer et al. (10) in melanoma cells. Therefore, we hypothesize that the role of RKIP in regulating cell proliferation is cell type specific via the regulation of different signal transduction pathways.

In our study, it is highly likely that RKIP regulates the proliferation and anchorage-independent growth of OVCA cells due to the regulation of the ERK signaling pathway. The Raf-1/MEK/ERK module is a ubiquitously expressed signaling cascade that controls proliferation and differentiation of many cell types. Recently, RKIP has been reported to be an inhibitory protein for the Raf-1/MEK/ERK module (12) and to possibly function as a rheostat that sets the sensitivity threshold for the activation of the Raf-1/MEK/ERK module pathway (15). Studies have shown that ERK1 and ERK2 were constitutively active in cell lines derived from epithelial and granulosa cell tumors, two distinct forms of OVCA, and that knockdown of ERK1/2 protein by RNA interference led to the complete suppression of tumor cell proliferation (22, 23). Consistent with this concept, we showed that in RKIP overexpressed SKOV-3 cells, phosphorylation of MEK and ERK was decreased compared with control cells, whereas knockdown of RKIP protein levels induced activation of MEK and ERK.

RKIP also inhibits nuclear factor-κB signaling by negatively modulating the activating phosphorylation of the inhibitor of nuclear factor-κB (IκB) kinase α and β via upstream kinases (24). Additionally, G protein signaling is facilitated by RKIP (25). G protein–coupled receptor (GPCR) kinase 2 is a critical negative feedback inhibitor of GPCRs. It was shown that RKIP is a physiologic inhibitor of GPCR kinase 2 (26). Specifically, after stimulation of GPCR, RKIP dissociates from Raf-1 and associates with GPCR kinase 2 and blocks its activity. This switch is triggered by the protein kinase C–dependent phosphorylation of the RKIP on Ser153 (27). Further experiments need to be done to determine if RKIP functions in OVCA, in part, by nuclear factor-κB and GPCR signaling pathways.

The in vitro invasion assay revealed that the overexpression of RKIP significantly suppressed their invasive ability, whereas down-regulation of RKIP promoted the invasive ability. Based on these findings, we did preliminary in vivo experiments using athymic nude mice. Experimental metastasis assay showed that ectopic RKIP overexpression significantly inhibited the implantation of OVCA cells.

The findings reported here suggest that in addition to its already described role in prostate cancer, melanoma, and breast cancer, RKIP also functions as metastasis suppressor gene in ovarian epithelial cancer by modifying several metastatic-associated phenotypes. Using orthotopically implanted ovarian tumor model, we confirmed that RKIP is able to suppress OVCA metastasis in vivo. Additionally, our data show an inverse correlation tendency between RKIP protein expression and malignant degree of ovarian epithelial tumors, suggesting that RKIP could be used as a marker in estimating the malignant degree of ovarian epithelial tumors.

Metastasis suppressor genes have been shown to suppress the growth of metastases without affecting the growth of the primary tumor (2). These genes differ from tumor suppressor genes, which suppress the growth of primary tumors. In our studies, overexpression or down-regulation of RKIP in OVCA cells affects not only the adhesive and invasive abilities, which were two of the key properties of the metastatic cascade, but also the tumorigenic properties, such as the proliferation and anchorage-independent growth. Thus, RKIP is a metastasis suppressor gene that also processes tumorigenic suppression properties in epithelial OVCA cells. However, to provide a potential valuable therapeutic strategy for OVCA metastasis, it is necessary to further investigate the underlying molecular mechanism of RKIP in suppressing OVCA metastasis.

### Materials and Methods

#### Cell Lines and Cell Culture

Human OVCA cell lines OVCAR-3, CAOV-3, and SKOV-3 were obtained from the American Type Culture Collection. High-metastatic human OVCA cell line HO-8910PM and the parental low-metastatic cell line HO-8910 were established by the Zhejiang Cancer Research Institute and purchased from Shanghai Institute of Cell Biology, Chinese Academy of Sciences. SKOV-3, HO-8910, and HO-8910PM cells were cultured in RPMI 1640 (Life Technologies, Inc.) containing 10% fetal bovine serum (FBS; Life Technologies); OVCAR-3 cells were grown in RPMI 1640 with 20% FBS; and CAOV-3 cells were grown in DMEM (Life Technologies) with 15% FBS.

#### Generation and Selection of Cells Stably Transfected with pcDNA3.1(+)-SsRKIP and pcDNA3.1(−)-AsRKIP

Transfection was done by using TRANSfection reagent (Tianwei) as recommended by the manufacturer’s instructions. SKOV-3 cells (5 × 10⁵) were plated onto six-well plates until 70% to 90% confluent before transfection. Cells were transfected with 4 μg of pcDNA3.1(+)-SsRKIP (i.e., sense RKIP vector) or pcDNA3.1(−)-AsRKIP (i.e., antisense RKIP vector), kindly provided by Dr. Evan T. Keller (University of Michigan, Ann Arbor, MI). Selection for the neomycin gene was initiated 48 h after transfection by adding 400 μg/mL of G418 (Life Technologies) to the supplemented culture medium. Resistant cell clones were isolated and expanded for further characterization. The empty vectors pcDNA3.1(+) and pcDNA3.1(−) were also transfected into SKOV-3 cells and served as negative controls.

#### Immunohistochemistry of Human Ovarian Epithelial Tumor Tissues and OVCA Cell Lines

Paraffin-embedded tissue sections from patients with benign ovarian epithelial tumor or epithelial OVCA were screened for RKIP protein expression by immunohistochemistry. Briefly,

### Table 3. Tumor Size of SKOV-3ip.Control and SKOV-3ip.Ss-RKIP

<table>
<thead>
<tr>
<th>Cell lines</th>
<th>Mean (mm³)</th>
<th>SD</th>
<th>P</th>
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<tbody>
<tr>
<td>Ss-RKIP-SKOV-3</td>
<td>3.493</td>
<td>2.927</td>
<td>0.000</td>
</tr>
<tr>
<td>Control</td>
<td>9.297</td>
<td>2.967</td>
<td></td>
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The table shows the paraffin-embedded tissue sections from patients with benign ovarian epithelial tumor or epithelial OVCA were screened for RKIP protein expression by immunohistochemistry. Briefly,
after blocking with normal goat serum at room temperature for 20 min, tissue sections were incubated with polyclonal rabbit RKIP antibody (1:160 dilution; Santa Cruz Biotechnology) overnight at 4°C. The detection was done using horseradish peroxidase and diaminobenzidine chromogen (Histostain kit, Zymed) according to the manufacturer’s instructions. Sections were then counterstained with hematoxylin, dehydrated, cleared, and mounted. Staining was scored as negative (0), weak (1), moderate (2), or strong (3) based on the amount of signal detection, as previously described (27).

High-metastatic HO-8910PM cells and the parental low-metastatic HO-8910 cell slides were fixed in methanol for 10 min, air dried, and stored at 4°C until used for immunohistochemistry, which was done with the polyclonal goat RKIP antibody (1:100 dilution; Santa Cruz Biotechnology) and the Histostain Bulk Goat IgG HRP SP Kit LAB-SA Detection System (Haoyang) according to the manufacturer’s instructions. The proportion of positively stained cells in each sample was determined by analyzing a total of at least 200 cells.

**Semiquantitative Reverse Transcription-PCR**

Total RNA was isolated from five OVCA cells using Trizol (Invitrogen) according to the manufacturer’s instructions and quantified by UV absorbance at 260 to 280 nm. Primer sequences were designed by Oligo 6 software and synthesized by TaKaRa Biotechnology Co. Ltd. The primer sequences were as follows: RKIP, 5′-CAATGACAT-CAGCAGTGGCACAGTC-3′ (forward) and 5′-CACAAGCT-CAATCCACACTGCCGCTG-3′ (reverse); glyceraldehyde-3-phosphate dehydrogenase, 5′-CTCAGACACCATGAGGA-AAGTGA-3′ (forward) and 5′-ATGATCTTGAAGCTGTGGT-CATA-3′ (reverse). One-Step RNA PCR kit (AMV; TaKaRa Biotechnology) was used to do reverse transcription-PCR. PCR products were fractionated on 1.5% agarose gel and analyzed with Quantity One-4.5.6 software (Bio-Rad). The results were normalized against GADPH and presented as target mRNA to GADPH ratio.

**Western Blot Analysis**

Whole-cell lysates were prepared by lysing the cells in ice-cold radioimmunoprecipitation assay buffer containing 150 mmol/L NaCl, 1% NP40, 0.5% deoxycholate, 0.1% SDS, 50 mmol/L Tris-HCl (pH 8.0), 1 mmol/L phenylmethylsulfonyl fluoride, 10 μg/mL leupeptin, and 100 μg/mL aprotinin for 45 min. After centrifugation, the supernatants were analyzed for protein concentration using the kit (Pierce Biochemicals). Total cell lysates (50 μg) were subjected to 12% SDS-PAGE gel electrophoresis and analyzed by Western blotting with goat polyclonal antibody for RKIP (1:200 dilution; Santa Cruz Biotechnology). The filters were stripped by incubating with stripping buffer containing 22.5 mmol/L Tris-HCl (pH 6.8), 2% SDS, and 100 mmol/L β-mercaptoethanol at 50°C for 30 min and then reblotted using mouse monoclonal antibody for β-actin at 1:4,000 dilution (Sigma). The results were visualized using chemiluminescent substrate kit (SuperSignal West Pico Trial kit, Pierce Biochemicals). Relative band intensities were assessed by densitometric analysis (Scion Image Analysis software program).

The phosphorylation status of endogenous MEK and ERK in SKOV-3 cells and in their stable transfectants was analyzed by Western blot with anti-phospho-MEK antibody (Cell Signaling Technology) to detect phosphorylated MEK or anti-phospho-ERK antibody (New England Biolabs) to detect phosphorylated ERK, visualized by chemiluminescent substrate kit. The filters were subsequently stripped and then reprobed with anti-MEK or anti-ERK antibodies (BD Biosciences) to detect both the phosphorylated and unphosphorylated forms of MEK and ERK, respectively.

**In vitro Cell Invasion Assay**

The invasiveness of five OVCA cell lines or different SKOV-3 stable cells was evaluated in 24-well Transwell chambers (Costar), as directed by the manufacturer. Briefly, the upper and lower culture compartments of each well were separated by polycarbonate membranes (8-μm pore size). The membranes were precoated with 50 μL (0.2 μg/μL) of Matrigel (Beijing Medical University, Beijing, China) dissolved and diluted in serum-free RPMI 1640 containing 0.1% bovine serum albumin (Sigma) were placed into the upper compartment of wells that were coated with the reconstituted matrix, and 600 μL of serum-free RPMI 1640 containing 0.1% bovine serum albumin were placed into the lower compartment. The Transwell chambers were incubated for 8 to 10 h at 37°C in a humidified 5% CO2 atmosphere. Cell penetration through the membrane was detected by staining the cells on the porous membrane with H&E staining and quantified by counting the numbers of cells that penetrated the membrane in five microscopic fields (at ×200 magnification) per filter. The experiment was repeated thrice.

**Cell Proliferation Assay**

Cell proliferation was measured with MTT assay. Briefly, SKOV-3 cells and the stable transfected clones were plated in 96-well plates at a density of 2 × 104 per well in 200 μL of RPMI 1640 with 5% charcoal-stripped FBS (Life Technologies). The cells were incubated at 37°C in a humidified 5% CO2 atmosphere for 1, 2, 3, 4, or 5 d, at which time the cells were subjected to 12% SDS-PAGE gel electrophoresis and analyzed by Western blotting with goat polyclonal antibody for RKIP (1:200 dilution; Santa Cruz Biotechnology). The filters were stripped by incubating with stripping buffer containing 22.5 mmol/L Tris-HCl (pH 6.8), 2% SDS, and 100 mmol/L β-mercaptoethanol at 50°C for 30 min and then reblotted using mouse monoclonal antibody for β-actin at 1:4,000 dilution (Sigma). The results were visualized using chemiluminescent substrate kit (SuperSignal West Pico Trial kit, Pierce Biochemicals). Relative band intensities were assessed by densitometric analysis (Scion Image Analysis software program).
were incubated for 40 min in the dark and analyzed by flow cytometry (FACSCalibur, BD Biosciences).

**Soft Agar Colony Formation Assay**

Assays of colony formation in soft agar were done using standard methods. Briefly, 1 mL underlayers consisting of 0.5% agar medium were prepared in six-well plates by combining 1 volume of 5% noble agar (Difco) with 9 volumes of RPMI 1640 with 10% FBS. Parental and transfected SKOV-3 clones were trypsinized, centrifuged, and resuspended in 0.3% agar medium (1 volume of 3% noble agar and 9 volumes of RPMI 1640 with 10% FBS); 1 × 10^4 cells were then plated onto the previously prepared underlayers. The cells were incubated at 37°C in a humidified 5% CO2 atmosphere for 14 d. Colonies were photographed and counted.

**In vitro Cell Adhesion Assay**

The adhesion of SKOV-3 cells stably transfected with sense or antisense RKIP was done using standard methods. Ninety-six-well plates were precoated with 25 μL (0.2 μg/μL) of Matrigel dissolved and diluted in serum-free RPMI 1640 overnight at 37°C. After blocking with 50 μL of 2% bovine serum albumin solution for 1 h at 37°C, 4 × 10^4 cells in 100 μL of serum-free RPMI 1640 containing 0.1% bovine serum albumin were placed into the wells precoated with the reconstituted matrix for 1 h at 37°C. The cells were washed with PBS and the MTT assay was done as described above. The data represent the average absorbance of eight wells in one experiment. The experiment was repeated twice with similar results.

**In vivo Metastasis Assays**

Female athymic BALB/c nu/nu mice, 4 to 6 wk old, were obtained from the Shanghai Institute of Material Medica, Chinese Academy of Sciences (Shanghai, China). All studies on mice were conducted in accordance with the NIH Guide for the Care and Use of Laboratory Animals. The study protocol was approved by the Shanghai Medical Experimental Animal Care Committee. Mice were injected with SKOV-3 cells that were stably transfected with either pcDNA3.1(+) (control SKOV-3) or pcDNA3.1(+)-SsRKIP (Ss-RKIP SKOV-3). To ensure that our results were not specific to any individual subclone of transfected cells, we injected pools of clones of each type of transfectant. The tumor cells were harvested from subconfluent cultures by a brief exposure to 0.25% trypsin and 0.02% EDTA. Trypsinization was stopped with medium containing 10% FBS. The cells were then washed twice in serum-free medium and resuspended in HBSS (serum-free). Only single-cell suspensions with >95% viability, as determined by trypan blue exclusion, were used for the injections. To produce tumors, 5 × 10^6 cells in 200 μL HBSS were injected intraperitoneally into nude mice. A total of five mice per group were used. The mice were sacrificed 20 d after inoculation with tumor cells. This time point was chosen because it gave a reproducible number of overt metastases without significant morbidity. A similar model has been used by other investigators (27). Then, the number and extent of overt metastases were quantified. The tumors were measured and their volumes were calculated using the formula for hemiellipsoids: \[ V = \frac{4}{3} \pi \sqrt{\frac{a^2}{2} \times \frac{b^2}{2} \times \frac{c^2}{2}} \] where \( a \), \( b \), and \( c \) are the dimensions of the tumor.

**Statistical Analysis**

The RKIP expression percentages across the tumor samples were compared using the Fisher’s exact test. The RKIP expression percentages in HO-8910 and HO-8910PM cells were compared using the Pearson’s \( \chi^2 \) test. We used the Spearman’s rank correlation test to analyze the significance of the association between RKIP expression level and the invasiveness of five OVCA cell lines or malignant degree of ovarian epithelial tumors. For the in vitro studies, single comparison was done using Student’s \( t \) test, and multiple comparisons were done using one-way ANOVA with Fisher’s protected least significant difference method for post hoc analysis. All statistical tests were two sided. For all tests, the level of significance was set at \( P < 0.05 \). Statistical analysis was done using the Statistical Package for the Social Sciences software version 10.0.

The nonparametric Wilcoxon rank sum test was used to analyze the differences in tumor size in the number of organ metastases per mouse between SKOV-3ip.Ss-RKIP and SKOV-3ip.Control clones.

**Disclosure of Potential Conflicts of Interest**

No potential conflicts of interest were disclosed.

**Acknowledgments**

We thank Dr. Evan T. Keller for providing pcDNA3.1(+) and pcDNA3.1(−) empty vectors and pcDNA3.1(+)SsRKIP and pcDNA3.1(−)AsRKIP plasmids and Hao Zhang for technical assistance.

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