

# Effects of Raf Kinase Inhibitor Protein Expression on Metastasis and Progression of Human Breast Cancer

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## Abstract

Raf kinase inhibitor protein (RKIP) has been shown to be a metastasis suppressor in many kinds of malignant tumors. But its function in breast cancer was not yet clarified completely. We detected RKIP expression in clinical samples of primary breast cancer, breast cancer metastases, and in different breast cancer cells. Compared with the normal breast epithelia, benign breast epithelia, or *in situ* ductal carcinoma, the expression level of RKIP is decreased in invasive carcinoma and significantly reduced or lost in the metastasis lymph node matched to the invasive carcinoma. To explore the potential role of RKIP in breast cancer metastasis, we studied the effect of RKIP on the malignant phenotypes of the breast cancer cells with ectopically overexpression or knockdown of RKIP. Cell proliferation, soft-agar colony formation, *in vitro* adhesion assay, invasion, and migration assays were done to examine the malignant phenotypes of the transfected cells. Consequently, RKIP has no effect on *in vitro* proliferation rate or colony-forming ability of MDA-MB-435 cells. *In vitro* cell invasion and migration assays indicated that the RKIP expression was inversely associated with the invasiveness of MDA-MB-435 cells. Consistent with these results, in the orthotopic murine models, we observed that overexpression of RKIP in breast cancer cells impaired invasiveness and metastasis, whereas down-regulation of RKIP expression promoted invasiveness and metastasis. These results indicate that *RKIP* is a metastasis suppressor gene of human breast cancer. (Mol Cancer Res 2009;7(6):832–40)

## Introduction

Distant metastases are the major cause of morbidity and mortality in women with breast cancer and represent the most serious challenge to therapeutic intervention. Metastasis is a complicated procedure involving lots of steps. Tumor cells must detach from the primary tumor, enter the circulation, and proliferate in the parenchyma of distant organs. A number of factors have been shown to be involved in this process, including proteases, adhesion molecules, motility factors, and angiogenic factors. Therefore, studies on the molecular mechanisms involved in breast cancer invasion and metastasis are vitally important in that they may lead to the identification of specific factors or genes that augment or suppress the metastasis or invasiveness of breast cancer cells.

Raf kinase inhibitor protein (RKIP) is one of these promising metastasis suppressors, which has various physiologic functions. The collective evidence indicates that RKIP regulates the activity and mediates the crosstalk between several important cellular signaling pathways, including the Raf/MAP/extracellular signal-regulated kinase kinase (MAPK)/ERK pathway (1), nuclear factor- $\kappa$ B (NF- $\kappa$ B) pathway (2), and G-protein pathway (3). A variety of ablative interventions suggest that reduced RKIP function may influence metastasis, angiogenesis, resistance to apoptosis, and genome integrity. It has been shown that reconstitution of RKIP expression prevented the invasion into the Matrigel and the metastasis in an orthotopic prostate cancer mouse model (1), but not the growth of the primary tumors, thus RKIP is a metastatic suppressor in prostate cancer (1, 4). Although RKIP is both metastatic suppressor and tumorigenic suppressor in ovarian cancer (5), for the RKIP affects not only the adhesive and invasive abilities of ovarian cancer, which were two of the key properties of the metastatic cascade, but also the tumorigenic properties, such as the proliferation and anchorage-independent growth. Although the reduced level of RKIP has been identified to correlate with breast cancer metastasis (6), the underline molecular mechanisms is still unclear. Breast cancer, prostate cancer, and ovarian carcinoma are endocrine-related and hormone-dependent tumors; thus, we hypothesize that the fundamental mechanisms regulating the metastatic colonization would be conserved in these hormone-dependent tumors. The purpose of the current study was to investigate whether RKIP functions as a metastatic or tumorigenic suppressor in breast cancer.

## Results

### *RKIP Expression in Human Breast Cancer*

Although it has been reported that RKIP was highly expressed in the original tumors than the metastatic tumors, it is

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**Note:** H.Z. Li and Y. Gao contributed equally to this work.

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unclear whether RKIP is a metastatic or tumorigenic suppressor in breast cancer. To investigate the potential relevance of RKIP expression to clinical breast cancer in Chinese population, we used an immunohistochemical approach to detect the RKIP in clinical samples of primary breast cancer and breast cancer metastases, which is consistent with the reported results (6). RKIP protein was found to be predominantly cytoplasmic distribution, partly in nuclear. Normal (84.85%) or benign breast epithelium (71.43%) and ductal carcinomas *in situ* (72.22%) showed strong staining. In contrast, invasive carcinoma was observed with markedly reduced staining. Totally, in node-positive primary tumors, RKIP expression was positive in 12 of 38 cases (31.58%), weakly positive in 22 of 38 cases (57.89%), and negative in 4 of 38 cases (10.53%). Whereas in the matched lymph node metastases obtained from the same patients, RKIP expression was considerably diminished. Four of 38 cases (10.53%) were positive, 16 of 38 cases (42.11%) were weak positive, and 18 of 38 cases (47.37%) RKIP was entirely absent. These data suggest that RKIP expression is significant decreased or lost in metastases ( $P \leq 0.001$ ; Table 1, Fig. 1).

Furthermore, we investigated the expression profile of RKIP in different breast cancer cells, to select the suitable one as the cell model to study the function of RKIP in the breast cancer. We detected the mRNA and protein level in metastatic MDA-MB-435 cells/MDA-MB-231 breast cancer cells, nonmetastatic T47D/MCF7 breast cancer cell lines, and breast epithelial cell HBL100. As shown in Fig. 2A and B, although there is no statistical significant ( $P > 0.05$ ) difference of RKIP expression for different breast cell lines, the metastatic cell line MDAMB435 has lower level of RKIP compared with other cell lines (Student's *t* test).

*Effect of RKIP Expression on Breast Cancer Metastasis*

To explore the role of RKIP in breast cancer cells, the human cell line MDA-MB-435 was used to reconstitute the expression of RKIP by stably overexpression (Ss-RKIP) or down-regulation (As-RKIP) of RKIP. Meanwhile, MDA-MB-435 cells were transfected with pcDNA 3.1(+) or (-) vector alone as negative control. The RKIP protein levels in these stable cells were determined by Western blot analysis. As shown in Fig. 2C and D, the cell lines Ss-RKIP-435 #14 and As-RKIP-435 #A1 were selected to perform further experiments.

Adhesion, invasion, and migration are key components of the metastatic cascade. Accordingly, *in vitro* adhesion, inva-

sion, and migration assays were done to evaluate the adhesion, invasion, and migration abilities of the parental MDA-MB-435 cells and their transfectants. As shown in Fig. 3, the ability of cell adhesion in the Ss-RKIP-435 cells was decreased compared with pcDNA3.1(+)-435 or parental MDA-MB-435, whereas the ability of cell adhesion in the Ss-RKIP-435 increased in the As-RKIP-435 cells ( $P < 0.001$ ). Furthermore, the shapes of cells were different after 60-minute culture on Matrigel Gel. Ss-RKIP-435 cells remained round with very little spreading after 60 minutes of culture, As-RKIP-435 cells had undergone spreading shape, and control groups had the two mixed shapes, which show different adhesion to Matrigel Gel. Overexpression of RKIP lead to an average of 52.5% decrease invasiveness compared with pcDNA3.1(+)-435, down-regulated RKIP expression cause an average of 113.2% increase in *in vitro* invasive ability compared with pcDNA3.1(-)-435 (Fig. 4). Furthermore, overexpression of RKIP lead to an average of 32.5% decreased migration compared with pcDNA3.1(+)-435. Conversely, down-regulated RKIP expression cause an average of 77.0% increased *in vitro* migration ability compared with pcDNA3.1(-)-435 (data not shown). These results suggest that RKIP expression is inversely associated with the adhesion, invasiveness, and migration ability of breast cancer cells *in vitro*.

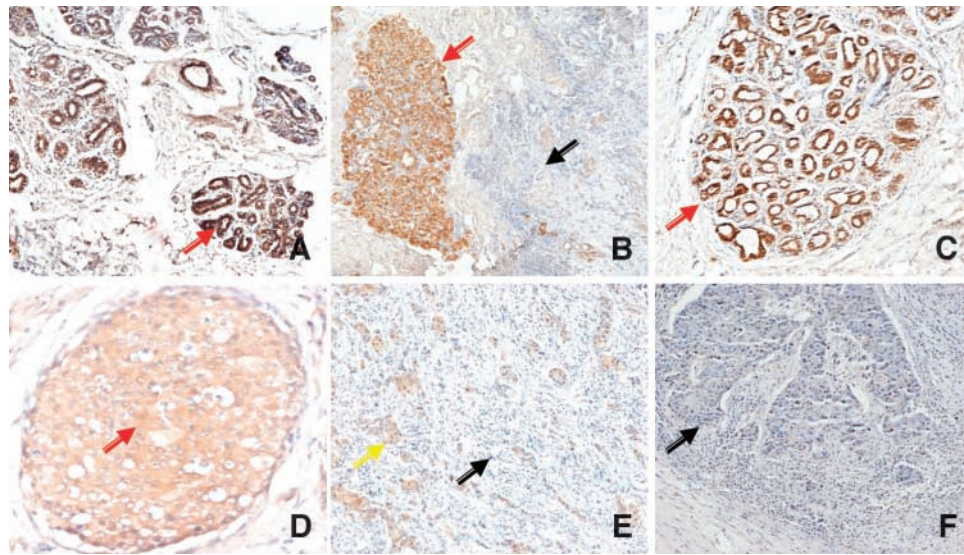
To examine the effect of RKIP-mediated metastasis suppression *in vivo*, the Ss-RKIP-435, As-RKIP-435, pcDNA3.1(+)-435, and pcDNA3.1(-)-435 cells were injected into the second or third mammary fat pad of nude mice. After the mean tumor diameter reached 1.0 to 1.5 cm, tumors were surgically removed and preserved in neutral-buffered formalin for histologic analysis. Five weeks later, mice were killed and examined for the presence of metastases in the lungs. Two independent experiments are reported in (Table 2). The average number of lung metastases of the mice injected with the Ss-RKIP-435 cells was significantly lower than that of the mice injected with pcDNA3.1(+)-435 cells. However, the average number of lung metastases of the mice injected with the As-RKIP-435 cells was significantly higher than that of mice injected with pcDNA3.1(-)-435 cells. These data indicate that ectopic expression of RKIP in the MDA-MB-435 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 breast cancer cell lines.

We also used immunohistochemistry to examine the RKIP expression levels of the primary and lung metastatic tumors in

**Table 1. Expression of RKIP in Human Breast Tissues**

Tissue	RKIP expression			Total n (%)
	Negative n (%)	Weak n (%)	Positive n (%)	
Normal tissue	3 (9.09)	2 (6.06)	28 (84.85)	33 (100)
Hyperplastic breast tissue	2 (14.29)	2 (14.29)	10 (71.43)	14 (100)
Ductal carcinoma <i>in situ</i>	0 (0)	5 (27.78)	13 (72.22)	18 (100)
Invasive ductal carcinomas (primary tumors)	4 (10.53)	22 (57.89)	12 (31.58)*	38 (100)
Lymph node metastases	18 (47.37)	16 (42.11)	4 (10.53) <sup>†</sup>	38 (100)

NOTE: Immunohistochemical staining of RKIP in human clinical tissue samples.  
 \* $P < 0.05$  compared with normal breast tissue, hyperplastic breast tissue and ductal carcinomas *in situ*.  
<sup>†</sup> $P = 0.001$  compared with primary invasive ductal breast carcinomas.

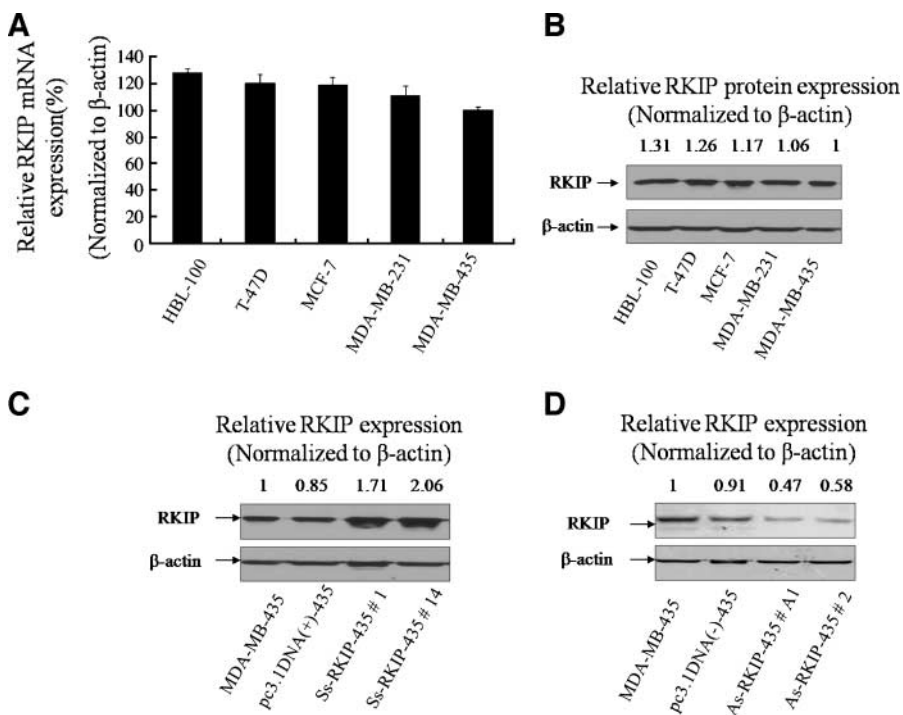


**FIGURE 1.** Immunohistochemical staining for RKIP in human breast tissues. Paraffin-embedded sections of human breast tissues were immunostained with a rabbit polyclonal antibody against RKIP, counterstained with hematoxylin, and photographed at  $\times 100$  magnification. **A.** Normal breast tissue, positive staining (red arrow). **B.** Para-carcinoma tissue, positive staining (red arrow); and carcinoma tissue, negative staining (black arrow). **C.** Breast hyperplasia tissue, positive staining (red arrow). **D.** Ductal carcinomas *in situ*, positive staining (red arrow). **E.** Invasive ductal carcinomas, weak (yellow arrow) and negative staining (black arrow). **F.** Invasive ductal carcinomas matched lymph node metastasis (from the same patient), negative staining (black arrow).

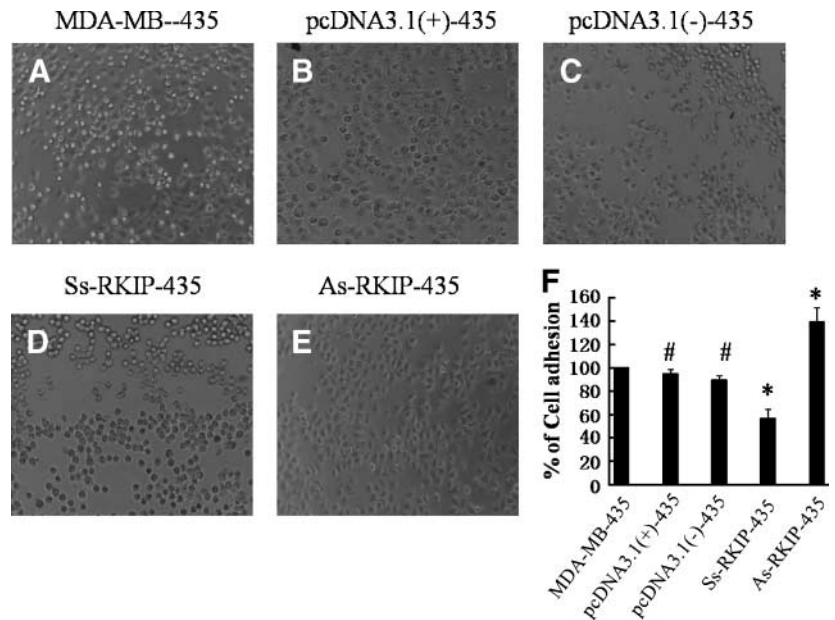
the mice. The primary tumors derived from the Ss-RKIP-435 cells showed higher expression levels of RKIP; however, the primary tumors derived from the As-RKIP-435 cells and lung metastases had little or no expression of RKIP, whereas the lung epithelia expressed RKIP, which suggests that RKIP expression was decreased in the metastases (Fig. 5).

#### Effect of RKIP Expression on Tumor Angiogenesis

Growth and progression of breast cancers are accompanied by increased neovascularization (angiogenesis). A variety of factors, including hypoxia and genetic changes in the tumor cells, contribute to increased production of angiogenic factors. Raf-1 activity mediates proliferation of endothelial cells, thereby



**FIGURE 2.** RKIP expression in different breast cancer cells and different clones of stably transfected MDA-MB-435 cells. Differential expression of RKIP in human breast cancer cells. **A.** RKIP mRNA expression in human breast cancer cells. The summary results from three separate experiments are presented in the panel. Bars, mean values of RKIP mRNA levels normalized to  $\beta$ -actin levels from three individual experiments. **B.** RKIP protein expression in five cell lines. RKIP band intensity was measured using densitometry and was normalized to  $\beta$ -actin levels. **C.** Parental MDA-MB-435 cells and clones stably overexpress RKIP. **D.** Parental MDA-MB-435 cells and clones stably knockdown RKIP. Band intensity was quantified using densitometry and was normalized to  $\beta$ -actin band intensity.



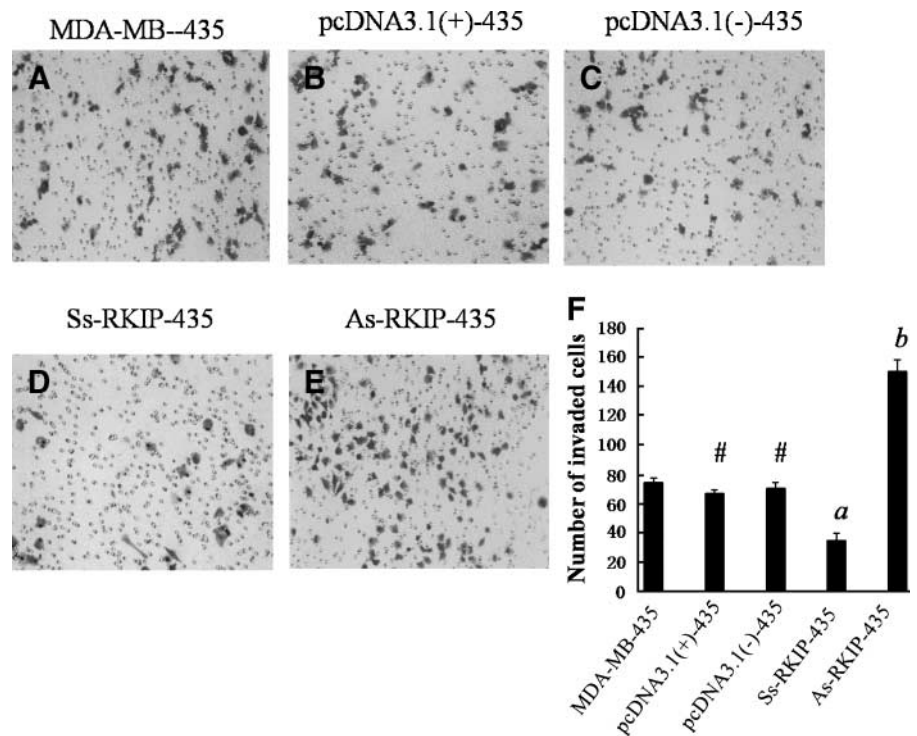
**FIGURE 3.** Effects of RKIP Expression on *in vitro* adhesion. RKIP suppresses *in vitro* cell adhesion of MDA-MB-435 cells. **A** to **E**. The shapes of MDA-MB-435 cells and their transfectants after 60-min culture on Matrigel Gel; Ss-RKIP-435 cells remained round with very little spreading; As-RKIP-435 cells had undergone spreading shape; control groups had the two mixed shapes (photographed at  $\times 100$  magnification). **F**. The adhesion of cells was measured by determined cell counts that adhered to Matrigel-coated 96-well plates after washing with PBS by MTS/PMS assay as described above. Columns, the percentage of adhesion compared with the control value and are the mean values of three experiments with sextuple samples; bars, SD. #,  $P > 0.05$  compared with the control-untransfected cells; \*,  $P < 0.05$  compared with the control-transfected cells.

promoting angiogenesis. Raf-1 silencing seems as a potential therapeutic strategy to inhibit brain tumor angiogenesis (7). RKIP is Raf-1 kinase inhibitory protein; thus, it may also influence angiogenesis. Accordingly, we examined the microvessel density within the primary tumors that developed in mice injected with human breast cancer cells. Morphometric analysis showed an increased density of CD31-positive microvasculature in tumors that developed from the As-RKIP-435 cells, whereas the Ss-RKIP-435 showed a decreased density

of CD31-positive microvasculature (Fig. 6). It suggests that RKIP has potential ability to inhibit the angiogenesis in breast cancer.

**Discussion**

Distant metastases are the major cause of morbidity and mortality in women with breast cancer. It is a highly organized process involving sequential steps, including tumor cell



**FIGURE 4.** Effects of RKIP expression on *in vitro* invasiveness. RKIP suppresses *in vitro* invasiveness ability of MDA-MB-435 cells. **A** to **E**. MDA-MB-435 cells and their transfectants that penetrated through the transwell chambers with Matrigel-coated and photographed at  $\times 100$  magnification. **F**. The *in vitro* invasiveness ability of MDA-MB-435 cells and their transfectants were measured by determining the number of Matrigel-coated cells that penetrated through the transwell chambers. Columns, mean values; bars, SD. a,  $P < 0.001$  compared with pcDNA3.1(+)-435; b,  $P < 0.001$  compared with pcDNA3.1(-)-435. #,  $P > 0.05$  compared with MDA-MB-435.

**Table 2. Metastatic Ability of MDA-MB-435 and their Transfectants *In vivo***

Cells	Tumor		Lung metastases	
	Incidence	Final tumor size, cm <sup>3</sup> mean $\pm$ SD	Mean number $\pm$ SD	Incidence/mice with primary tumor
MDA-MB-435	8/8	1.22 $\pm$ 0.11	4.50 $\pm$ 1.91	4/8
pc3.1(+)-435	7/8	1.18 $\pm$ 0.57	9.50 $\pm$ 7.72	4/7
pc3.1(-)-435	7/8	1.33 $\pm$ 0.37	6.67 $\pm$ 1.53	3/7
Ss-RKIP-435	6/8	1.38 $\pm$ 0.49*	1.50 $\pm$ 0.71 <sup>†</sup>	2/6 <sup>†</sup>
As-RKIP-435	8/8	1.24 $\pm$ 0.50*	13.8 $\pm$ 6.50 <sup>†</sup>	5/8 <sup>†</sup>

MDA-MB-435 cells and their transfectants were injected into the second mammary fat pads of female nude mice. Primary tumors were surgically removed when the mean tumor diameters reached 1.0 to 1.5 cm.

\* $P > 0.05$  compared with that for their empty transfectants.

<sup>†</sup>Five weeks later, mice were killed and lung metastases were counted.

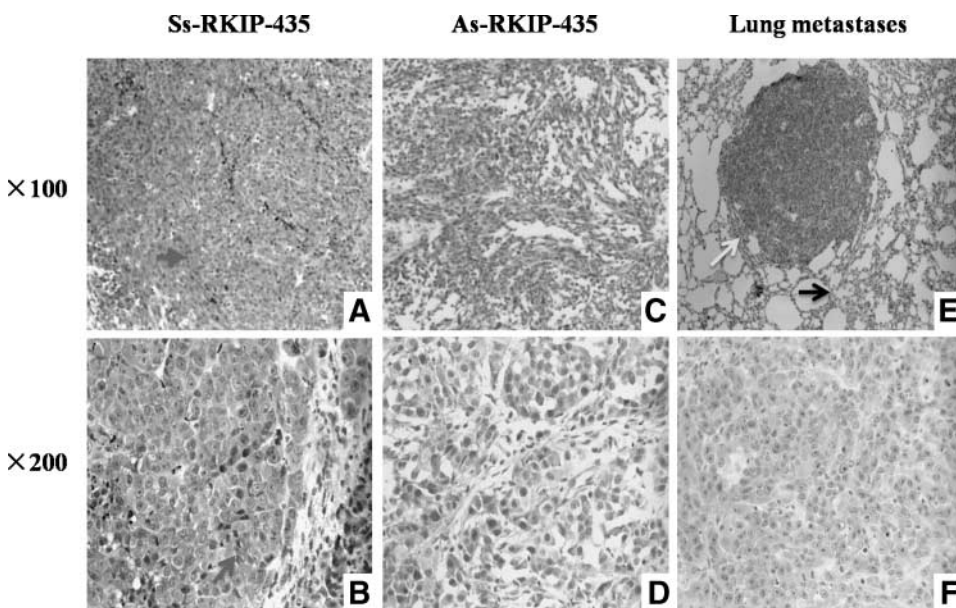
adhesion to microvessel endothelial cells and the subendothelial basement membrane mediated by extracellular matrix molecules and tumor cell migration, invasion toward basement membrane, and angiogenesis. Metastasis suppressor genes encode proteins that suppress the formation of overt metastases without affecting the growth rate of the primary tumor (8); it may inhibit early steps in metastatic colonization. Thus, identification of metastasis suppressor gene would provide the advantage to prevent the metastasis. A growing number of genes have been identified to be metastasis-suppressor genes, such as *Nm23* (9), *Drg-1* (*Differentiation-related gene*; ref. 10), *SSECKs* (*Src-suppressed C Kinase substrate*; ref. 11), *VDUP* (*Vitamin D3 up-regulated protein 1*; ref. 12), *Mitogen activated protein kinase kinase 4* (13), *RhoGDI2* (14), *Brms1* (15), *Kiss-1* (16), *Claudin-4* (17), and *Kail* (18).

Recently, *RKIP* was identified as a promising metastasis suppressor gene. In this contribution, we have shown that *RKIP* expression is associated with the suppression of breast cancer metastasis in the mice model and that loss of *RKIP* expression is associated with clinical metastasis. Using orthotopically implanted breast cancer model, we confirmed that *RKIP* expression

was lost in lung metastases. These findings indicate that *RKIP* is likely to be a metastasis suppressor gene in breast cancer.

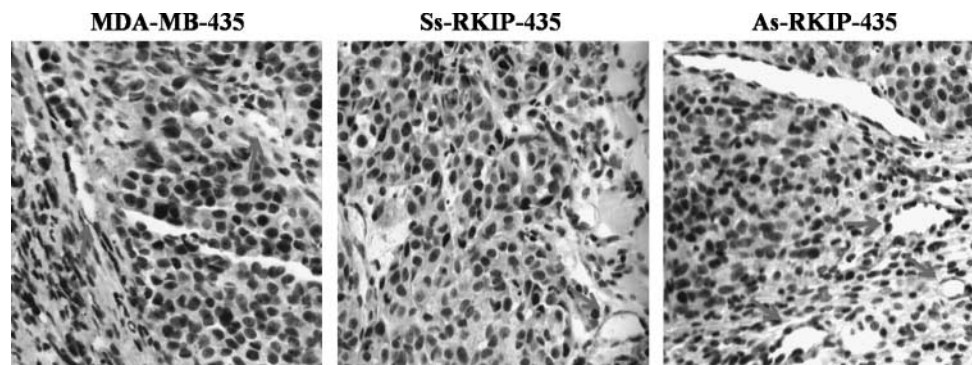
We showed that the expression of *RKIP* significantly decreased in invasive ductal carcinomas compared with normal breast tissue, breast hyperplasia tissue, ductal carcinomas *in situ*, whereas the result is not completely same with Hagan et al. (6). Furthermore, *RKIP* shows significantly diminished or lost in invasive ductal carcinomas matched lymph node metastases, suggesting an association between diminished *RKIP* expression and the occurrence of metastasis. The observation that the inverse correlation between *RKIP* expression and malignant degree of breast tumors suggests that decreased *RKIP* expression promotes breast cancer metastasis. *RKIP* possibly acts as a useful biomarker for identifying those patients with occult metastases.

Tumor invasion and tumor cell spreading involve tightly regulated interplay between the formation and loosening of adhesive contacts of tumor cells with the extracellular matrix. Although increased adhesion, invasion, and migration does not necessarily increase the metastatic ability of tumor cells, to some extent, these are the part of the metastatic cascade in a complex process in which many genes are involved. Accordingly,



**FIGURE 5.** Expression of *RKIP* in primary tumors and lung metastases of mice. **A.**  $\times 100$ ; **B.**  $\times 200$ . Primary tumors derived from Ss-RKIP-435 cells show higher levels of *RKIP* expression (brown; red arrowheads, individual cells). **C.**  $\times 100$ ; **D.**  $\times 200$ . Primary tumors derived from As-RKIP-435 cells; **E.**  $\times 100$ ; **F.**  $\times 200$ . Lung metastases (yellow arrowhead) had little or no expression of *RKIP*, whereas the lung epithelia expressed *RKIP* (black arrowhead).





**FIGURE 6.** Effects of RKIP expression on vascular angiogenesis. Vascular angiogenesis of mice implanted with MDA-MB-435, Ss-RKIP-435, or As-RKIP-435. Representative sections were stained for the antibody against the endothelial cell marker CD31 (brown) in primary tumors. Arrows, the CD31-positive cells ( $\times 200$ ).

our results showed an inverse correlation between RKIP expression and the *in vitro* cell adhesion to basement membrane, invasion, or migration ability. The contribution of a particular molecule to metastasis can only be convincingly clarified by *in vivo* studies. Thus, to examine whether RKIP expression is associated with cancer cell metastasis *in vivo*, the nude mice were sacrificed to set up the primary and metastatic breast cancer animal model. The data show that overexpression of RKIP in breast cancer cells inhibited the development of lung metastases. In contrast, knockdown of RKIP promoted the lung metastasis.

To examine whether modulation of RKIP expression influenced the tumorigenic properties of the breast cancer cells, we tested *in vitro* cell proliferation and anchorage-independent growth of Ss-RKIP-435, As-RKIP-435, or control MDA-MB-435 cells. There were no significant differences in *in vitro* proliferation rates or colony-forming abilities (data not shown). These results were further confirmed *in vivo*. In the orthotopic nude mice models, we also measured the primary tumor size, which had no significant difference between the control and the Ss-RKIP cells, or the control and the As-RKIP-435 cells (data not shown). These data suggest that RKIP has no effect on primary tumorigenic properties of human breast cancer cells. Metastasis suppressor genes have been defined to suppress the growth of metastases without affecting the growth of the primary tumor; thus, our study provides important mechanistic information that RKIP is the metastatic suppressor in breast cancer.

Tumor growth, progression, and metastasis require angiogenesis. Tumor spread to distant sites is also dependent on access to the vasculature (19). Our data suggest that decreased expression of RKIP promotes angiogenesis; however, increased expression of RKIP suppress angiogenesis in the tumor tissue. Accordingly, one factor of decreased RKIP promotes metastasis is that increased number of blood vessels available for invasion, the higher the count of microvessels and the larger the surface area of these vessels, the higher the probability that tumor cells will enter the circulation (19, 20). In summary, our study shows that RKIP is a metastasis suppressor of breast cancer. The decreased RKIP expression has been correlated with poor clinical outcome in breast cancers, increased neovascularization and adhesion to the extracellular matrix, increased motility, invasion, and metastasis.

Metastasis suppressors seem to regulate selectively how cells respond to exogenous signals, by affecting signaling cascades that regulate downstream gene expression. RKIP is an inhibitor of Raf-mediated signaling (1). RKIP also inhibits

NF- $\kappa$ B signaling by negatively modulating the activating phosphorylation of the inhibitor of NF- $\kappa$ B kinase  $\alpha$  and inhibitor of NF- $\kappa$ B kinase  $\beta$  via upstream kinases (2). Additionally, G-protein signaling has been shown to be facilitated by RKIP (3). These pathways are involved in the regulation of many fundamental cellular processes, including proliferation, differentiation, survival, and cell death. RKIP levels change may have effect on these processes. Further experiments need to be done to determine if RKIP functions in breast cancer, in part, by Raf/MEK/ERK, NF- $\kappa$ B, and G-protein signaling pathways.

Despite many of the unresolved questions, RKIP remains a promising molecular target for the treatment of metastatic disease and has shown great promise as a prognostic indicator for several cancers. Devasis (21) show that the metastatic potential of tumor cells could be prevented by chemotherapy-triggered induction of RKIP. Some cancer cells, when subjected to an apoptotic insult, have the ability to up-regulate RKIP expression, and become sensitive to apoptosis. Reexpression of RKIP expression by micrometastatic tumor cells may have therapeutic effects on cancer progression. Metastasis suppressors are usually transcriptionally silenced, rather than mutated, in aggressive tumors. Thus, if well-tolerated agents could be identified that elevate metastasis suppressor expression and therefore function in micrometastatic tumor cells, this strategy might constitute a new therapeutic endeavor.

Although breast cancer, prostate cancer, and ovarian carcinoma all are endocrine-related and hormone-dependent tumors, the function of RKIP is variable. For example, RKIP acts as a metastatic suppressor in prostate cancer and breast cancer, but both metastatic suppressor and tumorigenic suppressor in ovarian cancer (5). It is necessary to further investigate the underlying molecular mechanism of RKIP in these hormone-related tumors.

## Materials and Methods

### Immunohistochemistry of Human Tissue

Paraffin-embedded tissue from normal or tumor region were sectioned, deparaffinized, and rehydrated through graded alcohol to distilled water. After blocking with normal goat serum at room temperature for 20 min, tissue sections were incubated with polyclonal rabbit RKIP antibody (1:400 dilution; Upstate Biotechnology) overnight at 4°C, and then detected with horseradish peroxidase and diaminobenzidine chromogen (Histostain

kit; Zymed). Sections were then counterstained with hematoxylin, dehydrated, cleared, and mounted. A score was established corresponding to the sum of: (a) the percentage of positive cells; 0, negative; 1, <25% positive cells; 2, 26% to 50% positive cells; and 3, >50% positive cells; and (b) the staining intensity (0, negative; 1, weak; 2, moderate; 3, strong). The (a) sum for the assigned values of the positive cell percentage and (b) the staining intensity was 6 or <6. Scores between 0 and 2 were regarded as negative, scores of 3 and 4 as weakly positive, and scores of 5 and 6 as positive (22).

#### *Cell Lines and Culture Conditions*

Human breast carcinoma cell lines, MCF-7, T-47D, MDA-MB-231, and MDA-MB-435 were obtained from the American Type Culture Collection. HBL-100, human breast epithelial cell line, was purchased from Shanghai Institute of Cell Biology, Chinese Academy of Sciences. Cell lines were maintained in DMEM (Life Technologies, Inc.), supplemented with 10% fetal bovine serum (FBS; Life Technologies, Inc.).

#### *Reverse Transcription and Real-Time Reverse Transcription-PCR*

Total RNA was isolated from five cell lines using TRIZOL (Invitrogen) according to the manufacturer's instructions and quantified by UV absorbance at 260 to 280 nm. Total RNA was used for cDNA synthesis using the BioRT cDNA First-Strand Synthesis kit (Bioer Technology Co., Ltd). cDNA from each sample was used as real-time PCR template. We used oligonucleotide primers synthesized by TaKaRa Biotechnology Co., Ltd (China). Reverse transcription-PCR of  $\beta$ -actin transcripts was used as an internal control to ensure equal loading of samples. The primer sequences were as follows: RKIP, 5'-CAATGACATCAGCAGTGGCACAGTC-3' (forward) and 5'-CACAAGTCATCCACTCGGCTG-3' (reverse). Power SYBR Green PCR Master Mix (Applied Biosystems) was used as amplification reaction mixture according to the manufacturer's instructions. Briefly, hold 95°C for 10 min to activate AmpliTaq Gold Polymerase. PCR reactions were subjected to 40 cycles of 95°C for 15 s, 60°C for 1 min.

#### *Stable Gene Transfection*

Transfection was done using FuGENE HD Transfection Reagent (Roche) as recommended by the manufacturer's instructions. Briefly, MDA-MB-435 ( $8 \times 10^5$  cells, in a log-growth phase) were plated onto 6-well plates until 80% to 90% confluence before transfection. Cells were transfected with the transfection complex containing 2  $\mu$ g 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 800  $\mu$ g/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 MDA-MB-435 cells and served as negative controls.

#### *Western Blot Analysis*

Whole cell lysates were prepared by lysing the cells in ice-cold radioimmunoprecipitation assay buffer containing

150 mmol/L NaCl, 1% Nonidet P-40, 0.5% deoxycholate, 0.1% SDS, 50 mmol/L Tris-HCl (pH 8.0), 1 mmol/L phenylmethylsulfonyl fluoride, 10  $\mu$ g/mL leupeptin, and 100  $\mu$ g/mL aprotinin for 45 min. Total cell lysates (40  $\mu$ g) were subjected to 12% SDS-PAGE gels electrophoresis and analyzed by Western Blotting with rabbit polyclonal antibody for RKIP (1: 1,000 dilution; Upstate Biotechnology). The filters were stripped and then reprobed using mouse monoclonal antibody for  $\beta$ -actin at 1: 6,000 dilution (Sigma). The results were visualized using chemiluminescent substrate kit (SuperSignal Westpico Trial kit). Relative band intensities were assessed by densitometric analysis (TotalLab v2.01 Analysis software program).

#### *Cell Proliferation Assay*

Parental cells (MDA-MB-435 cells) and their transfectants were plated in 96-well plates at a density of 1,000 cells per well in 200  $\mu$ L of DMEM with 5% FBS. The cells were incubated at 37°C in a humidified 5% CO<sub>2</sub> atmosphere for 2, 4, 6, or 8 d, at which time 20  $\mu$ L of combined MTS/PMS solution (Promega) was added per well. After incubation for 2 h at 37°C, we measured the absorbance of each well at 490 nm by using an ELISA plate reader. Data represent the average absorbance of six wells in one experiment. The experiment was repeated thrice with similar results.

#### *Soft-Agar Colony Formation Assay*

Assays of colony formation in soft agar were done using standard methods. Briefly, parental and transfected MDA-MB-435 cells ( $1 \times 10^4$  cells per well) were suspended in 0.3% Noble agar and were plated onto a layer of 0.6% Nobleagar in DMEM containing 10% FBS in 6-well tissue culture plates (Corning). The agar containing cells was allowed to solidify overnight at 37°C in 5% CO<sub>2</sub> humidified atmosphere. Additional DMEM containing 10% FBS was overlaid on the agar and the cells allowed growing undisturbed for 2 wk. Visible colonies (>50 cells) were counted with the aid of a microscope.

#### *In vitro Cell Adhesion Assay*

To compare the adhesion ability of Parental and transfected MDA-MB-435 cells attach to the basement membrane, we did adhesion assays using standard methods. Briefly, 96-well plates were precoated with 20  $\mu$ L of Matrigel (BD Biosciences) 1 h at 37°C, and the plates were washed with PBS twice. After blocking with 50  $\mu$ L of 2% bovine serum albumin (BSA) solution for 1 h at 37°C,  $2 \times 10^5$  cells in 100  $\mu$ L of serum-free DMEM containing 0.1% BSA were placed into the wells precoated with the reconstituted matrix for 1 h at 37°C. The cells were washed with PBS and the MTS/PMS assay was done as described above. Each data represents the average absorbance of six wells in one experiment. The experiment was repeated thrice with similar results.

#### *In vitro Cell Invasion Assay*

The invasiveness of MDA-MB-435 cells stably transfected with control, sense, or antisense plasmids was evaluated in 24-well transwell chambers (Costar), as directed by the manufacturer. Briefly, the upper and lower culture compartments of each well are separated by polycarbonate membranes (8  $\mu$ m

pore size). The membranes were precoated with 20  $\mu$ L Matrigel (BD Biosciences) at 37°C for 1 h. The lower chambers of the 24-well plate were filled with 600  $\mu$ L DMEM containing 10% FBS;  $5 \times 10^4$  cells in 100  $\mu$ L of serum-free DMEM containing 0.1% BSA (Sigma) was placed into the upper compartment of wells that were coated with the reconstituted matrix. The transwell chambers were incubated at 37°C in 5% CO<sub>2</sub> humidified atmosphere for 48 h. The cells that had not invaded were removed from the upper face of the filters using cotton swabs. The cells that had invaded to the lower surface of the filters were fixed and stained in 0.1% crystal violet (23), and quantified by counting the numbers of cells that penetrated the membrane in five microscopic fields (at  $\times 100$  magnification) per filter. The experiment was repeated thrice.

#### *In vitro Cell Migration assay*

A transwell chamber assay was used. The lower chambers of the 24-well plate were filled with 600  $\mu$ L DMEM containing 10% FBS, and  $5 \times 10^4$  cells in 100  $\mu$ L of serum-free DMEM containing 0.1% BSA (Sigma) was placed into the upper compartment of wells that did not contain Matrigel Gel. The transwell chambers were incubated at 37°C in 5% CO<sub>2</sub> humidified atmosphere for 24 h. Further staining and recording of results was done as mentioned above in invasion assay.

#### *Mice*

Female athymic BALB/c nu/nu mice, 4- to 6-wk-old, were obtained from Shanghai Institute of Material Medical, 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.

#### *Spontaneous Metastasis Assay*

Mice were injected with Parental and transfected MDA-MB-435 cells ( $n = 5$ /group, 2 independent experiments), respectively. Immediately before injection, cells at 80% to 90% confluence were detached with 0.25% trypsin and 0.02% EDTA, washed with chilled HBSS (Invitrogen), counted, and resuspended in ice-cold HBSS to a final concentration of  $1 \times 10^7$  cells/mL. Only single-cell suspensions with >95% viability, as determined by trypan blue exclusion, were used for the injections. Cells ( $2.0 \times 10^6$  cells/200  $\mu$ L) were injected into the second or third mammary fat pad using a 29-gg needle affixed to a 1-cc tuberculin syringe.

Tumor size was measured weekly and mean tumor volumes calculated by taking the square root of the product of orthogonal measurements. After the mean tumor diameter reached 1.0 to 1.5 cm, tumors were surgically removed under anesthesia and the wounds closed with sterile stainless steel clips. Tumor tissue was preserved in neutral-buffered formalin for histologic analysis. Five weeks later, mice were killed and examined for the presence of metastasis. Lungs were removed, rinsed in saline, and fixed in diluted Bouin's solution (20% Bouin's fixative in neutral buffered formalin) before quantification of visible surface metastasis as described (24).

#### *Assessment of Angiogenesis*

To quantify vascularity in the primary tumors in mice, we did immunohistochemistry to examine the microvessel density within the primary tumors that developed in mice injected with human breast cancer cells. Briefly, the primary tumors were fixed in 10% formalin, embedded in paraffin, and sectioned, deparaffinized, and rehydrated through graded alcohol to distilled water, then stained for CD31 with a rabbit polyclonal anti-CD31 antibody (ab28365; Abcam) using standard immunohistochemistry techniques, as described above. Vascularity was determined by the number of vessels per field counted in the area of highest vascular density at  $\times 200$  magnification. Recommended guidelines were followed (25). Single endothelial cells, endothelial cell clusters, and microvessels in the tumor clearly separated from adjacent microvessels were counted. Peritumoral vascularity and vascularity in areas of necrosis were not scored. Branching structures were counted as a single vessel. The presence of lumen or erythrocytes in the lumen was not required to classify a structure as a vessel. If the vascularity was uniform, microvessels in three fields were counted and averaged. If the vascularity in different fields was not uniform, up to 10 fields were counted, and the 3 highest counts were averaged.

#### *Statistical Analysis*

The RKIP expression percentages across the tumor samples were compared using the  $\chi^2$  test. 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. The nonparametric Wilcoxon rank sum test was used to analyze the differences in tumor size and the number of organ metastases per mouse between these groups. All statistical tests were two sided. For all tests, the level of significance was set at a *P* value of <0.05. Statistical analysis was done using the Statistical Package for the Social Sciences software version 11.5.

#### **Disclosure of Potential Conflicts of Interest**

No potential conflicts of interest were disclosed.

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