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RKIP INHIBITS THE MIGRATION AND INVASION OF HUMAN PROSTATE CANCER PC-3M CELLS THROUGH REGULATION OF EXTRACELLULAR MATRIX

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Raf kinase inhibitor protein (RKIP) plays a pivotal role in several intracellular signaling cascades and has been implicated as a metastasis suppressor in multiple cancer cells including prostate cancer cells, but the mechanism is not very clear. In this study, we investigated the effect of RKIP on cell proliferation, migration and invasion using human prostate cancer PC-3M cells as a model system. Our results indicate that RKIP does not effect cell proliferation in PC-3M cells, but inhibits both cell migration and cell invasion. In association with this inhibitory effect, RKIP down-regulates matrix metalloproteinases (MMP-2 and MMP-9), cathepsin B and urinary plasminogen activator (uPA). Also RKIP has the ability to regulate the expression of E-cadherin. But ectopic expression of RKIP does not affect the level of the Snail protein. As it has been indicated here, RKIP inhibits the migration and invasion ability of human prostate cancer cells through regulation of the extracellular matrix. These findings provide new mechanistic insight how RKIP suppresses metastasis *in vitro*.

***Keywords*: RKIP, migration, invasion, extracellular matrix.**

INTRODUCTION

Raf Kinase Inhibitory Protein (RKIP), a member of the phosphatidylethanolamine-binding protein family, plays a pivotal modulatory role in MAPK G-protein-coupled receptor and NF-κB signaling pathways [1]. RKIP regulates a number of cellular processes including membrane biosynthesis, spermatogenesis, neural development, apoptosis and cancer metastasis. Subsequent studies revealed that RKIP was implicated as a suppressor of tumor cell metastasis in prostate cancer, breast cancer, melanoma, hepatocellular carcinoma, ovarian cancer and colorectal cancer cells [2–7]. Restoration of RKIP expression inhibits prostate cancer metastasis in a murine model [2]. RKIP is also known as a good prognostic marker of pathogenesis and its expression correlates with the overall and disease free survival of human prostate cancer [8] and colorectal cancer [5].

In prostate cancer cells, RKIP does not prevent the growth of primary tumors. But in ovarian cancer, RKIP affects not only the adhesive and invasive abilities of the cells, but also cell proliferation and anchorage-independent growth. Apart from these, there are some reports suggesting that RKIP promotes cell migration by down-regulating E-cadherin and up-regulating β1 integrin [9, 10]. Underlining the molecular mechanisms by which RKIP prevents the adhesive and invasive abilities of prostate cancer would be helpful for understanding these differences.

To reveal the mechanism of tumor metastasis suppression by RKIP, we designed this study to determine the effect of RKIP on the cell proliferation, migration and invasion. The RKIP overexpressing PC-3M cell model was constructed and the molecular mechanisms involving RKIP were explored. We found that migration and invasion, but not the proliferation of PC-3M cells was inhibited by RKIP. MMP-2, MMP-9, uPA and cathepsin B, which play key roles in tumor invasion and metastasis, were down-regulated by RKIP. In addition, RKIP is able to up-regulate the level of E-cadherin, an important adherents' junction protein. Our results may facilitate to better understanding of the molecular mechanisms by which RKIP prevents the migratory and invasive abilities of prostate cancer.

EXPERIMENTAL

Cell lines and cell culture. The prostate cell lines PC-3M (gift from Dr. Rouli Zhou) were cultured in DMEM ("Invitrogen") and 10% fetal bovine serum ("HyClone") in a 5% CO_2 atmosphere at 37°C.

Plasmid constructs. Human RKIP cDNA was PCR amplified for in-frame cloning into pcDNA3.1 (–)

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Abbreviations: MMP – matrix metalloproteinase; RKIP – Raf Kinase Inhibitory Protein; uPA – urinary plasminogen activator.

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Primer sequences

Target	Primers	Product length (bp)
RKIP	Forward: 5'-TATGCCGGTGGACCTCAG-3' Reverse: 5'-CCTACTTCCCAGACAG-3'	600
MMP-2	Forward: 5'-AACCCTCAGAGCCACCCCTA-3' Reverse: 5'-GTGCATACAAAGCAAACTGC-3'	287
MMP-9	Forward: 5'-CGGGACGGCAATGCTGATG-3' Reverse: 5'-CGCCACGAGGAACAAACTGT-3'	378
Cathepsin B	Forward: 5'-ACAGTGTCCCACCATCAAAG-3' Reverse: 5'-CACCATTACAGCCGTCCC-3'	185
uPA	Forward: 5'-CCCCACTACTACGGCTCT-3' Reverse: 5'-GCTATTGTCGTTCACCCTG-3'	409
β-actin	Forward: 5'-GAGCTACGAGCTGCCTGACG-3' Reverse: 5'-CCTAGAAGCATTTGCGGTGG-3'	416

vector using the primers 5'-TTTTCTCGAGTATGCCGGTGGACCTCAG-3' and 3'-CAAGCGAATTCCCTACTTCCCAGACAG-5'. The expression plasmid pcDNA-RKIP was constructed using XhoI and EcoRI. Sequence analysis showed that the subcloned RKIP cDNA had a 100% homology to published sequence for RKIP cDNA. The empty pcDNA3.1 (–) vector without the RKIP inserting was used as a negative control in subsequent experiments.

Generation and selection of cells stably transfected with pcDNA3.1(–)-RKIP. PC-3M cells stably expressing RKIP were obtained by transfection using Lipofectamine TM2000 ("Invitrogen", USA) according to the manufacturer's instruction. Twenty-four hours after the transfection, the cells were transferred into the selection medium containing neomycin G418 (400 μg/mL) for four weeks. After selection with G418, overexpressing RKIP vectors or a control empty vector were isolated, pooled, and used for subsequent gene expression analysis by RT-PCR and Western blotting.

MTT assay. Cells were seeded in 96-well plates (5000 cells/well).The 3-(4, 5-dimethylthiazol-2-yl)-2, 5-diphenyltetrazolium bromide (MTT) assay was used in the assay of the cell viability as previously described [11]. The experiments were carried out independently at least three times.

Cell cycle assay. The cells were collected and fixed by 70% ethanol at –20°C overnight and then washed twice with cold PBS. Then the cells were suspended in PBS with propidium iodide (10 mg/mL; "Sigma", USA) and RNAse AI (1 mg/mL) and incubated at 37°C for 1h. Distribution of cells with different DNA content was determined using the FACSVantage SE System ("BD", USA).

***In vitro* wound-healing assay.** PC-3M cells (3×10^5 cells/mL) were cultured in 35-mm dishes until confluent. The monolayer of PC-3M cells was scratched with the tip of a pipette in order to wound the monolayer. Photomicrographs at 10×-objective magnification were taken at time points of 0, 4 8, 12, and 24 h. All samples were examined in triplicate.

In vitro migration and invasion assay. For transwell migration assays, 2.5×10^4 to 5×10^4 cells were plated in the top chamber with the non-coated membrane (24-well insert; pore size, 8 μm; "Corning Inc."). For invasion assays, 1.25×10^5cells were plated in the top chamber with Matrigel (1mg/ml; "Sigma")-coated

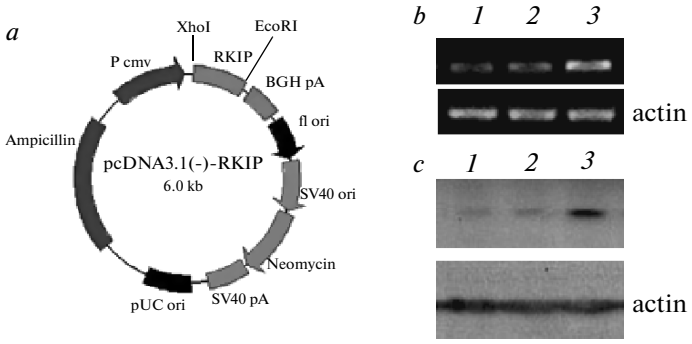

Fig. 1. Ectopic expression of RKIP in PC-3M cells. *a* – RKIP expression vectors; *b* – RT-PCR analysis; *c* – Western blotting: the cell lysate proteins were separated by SDS-PAGE and analyzed with antibodies against RKIP. Here: *1* – PC-3M cells, *2* – vector control, *3* – RKIP ectopic expression.

membrane (24-well insert; pore size, 8 μm; "Corning Inc."). In both assays, cells were plated in medium with 5% serum; and medium containing 15% serum was used as a chemo-attractant in the lower chamber. The cells were incubated for 6 h for migration assay and 16 h for invasion assay at 37°C in a 5% CO_2 atmosphere. Cells that did not migrate or invade through the pores were removed by a cotton swab. Cells on the lower surface of the membrane were stained with hematoxylin and manually counted under a microscope. Each assay was performed in triplicate.

RT-PCR. Total RNA was extracted using the TRIZOL reagent ("Invitrogen", USA) according to the manufacturer's instruction. For reverse transcription, 2μg of RNA were annealed to oligo(dT) at 65°C for 5 min and cooled at room temperature. Reverse transcriptase and dNTPs were added to the RNA-oligo(dT) mixture and the reaction was performed at 42°C for 1 h. Primers used are listed in the table. β-actin was used as the loading control. PCR reaction was stopped after 25 cycles.

Western blotting. Cells were harvested and lysed in the buffer containing 25 mM Tris-HCl, pH 7.5, 150 mM NaCl, 2 mM EDTA, 10% glycerol, 10 mM glycerophosphate, 5 mM sodium pyrophoshate, 5 mM NaF, 1 mM Na_3VO_4, 0.5% Triton X-100, with a freshly added protease inhibitors cocktail ("Ameresco"). 40 μg of protein for each sample were denatured at 100°C for 10 min and loaded on the 10% SDS–PAGE. After electrophoresis, proteins were transferred to a PVDF-membrane, and probed by corresponding antibodies. Antibodies against RKIP, E-cadherin, phospho-CREB (Ser133) (CREB protein which is phosphorylatd at Ser133) were obtained from the Cell Signaling Technology ("Cell Signaling", USA). Anti-uPA, anti-p65, anti-p50 and anti-actin antibodies were from Santa Cruz Biotechnology ("Santa Cruz", USA). Anti-cathepsin B and anti-Snail antibodies were from Abcam ("Abcam", GB).The protein signals were detected by the enhanced chemiluminescence reaction system according to the manufacturer's recommendations ("Amersham Biosciences", USA).

Zymography. Cells were incubated in serum-free medium overnight. The supernatant was collected and concentrated with an Amicon Ultra-4 centrifugal device ("Millipore"). Proteins in a sample (2 μg of protein) were resolved by electrophoresis under non-denaturing conditions in a 10% polyacrylamide gel containing 0.1% gelatin ("Invitrogen") as a substrate. After electrophoresis, the gel was washed twice with 2.5% Triton X-100 (v/v) for 30 min, followed by a brief rinsing in 50 mM Tris-HCl, pH 7.6, containing 5 mM $CaCl_2$, 1 μM $ZnCl_2$. The gel was then incubated at 37°C for 16 h in the same buffer. Then the gel was stained with 0.1% Coomassie Brilliant Blue R250 ("Sigma") followed by destaining with a water solution of 10% acetic acid and 45% methanol. Enzyme-containing regions were observed as white bands against a blue background. Zones of enzymatic activity were seen as negatively stained bands.

Statistical analysis. Data were expressed as mean value ± SEM. Differences between experimental groups were assessed using the Student's *t*-test; $P < 0.05$ (*), $P < 0.01$ (**) were considered as statistically significant.

RESULTS

The effect of RKIP on cell proliferation, migration and invasion ability of PC-3M cells

In order to investigate the function of RKIP, human RKIP cDNA was PCR amplified for in-frame cloning into the pcDNA3.1(–) plasmid (fig. 1*a*). As RKIP was weakly expressed in the PC-3M cells, we stably transfected the human prostate cancer cell line PC-3M with the pcDNA3-neo vector containing a full-length RKIP cDNA or with the pcDNA3.1(–) plasmid serving as a control (see "Experimental"). After selection with G418, a RKIP overexpressing vector or a control empty vector were isolated, pooled, and used for subsequent gene expression and functional studies. The ectopic expression of RKIP was con-

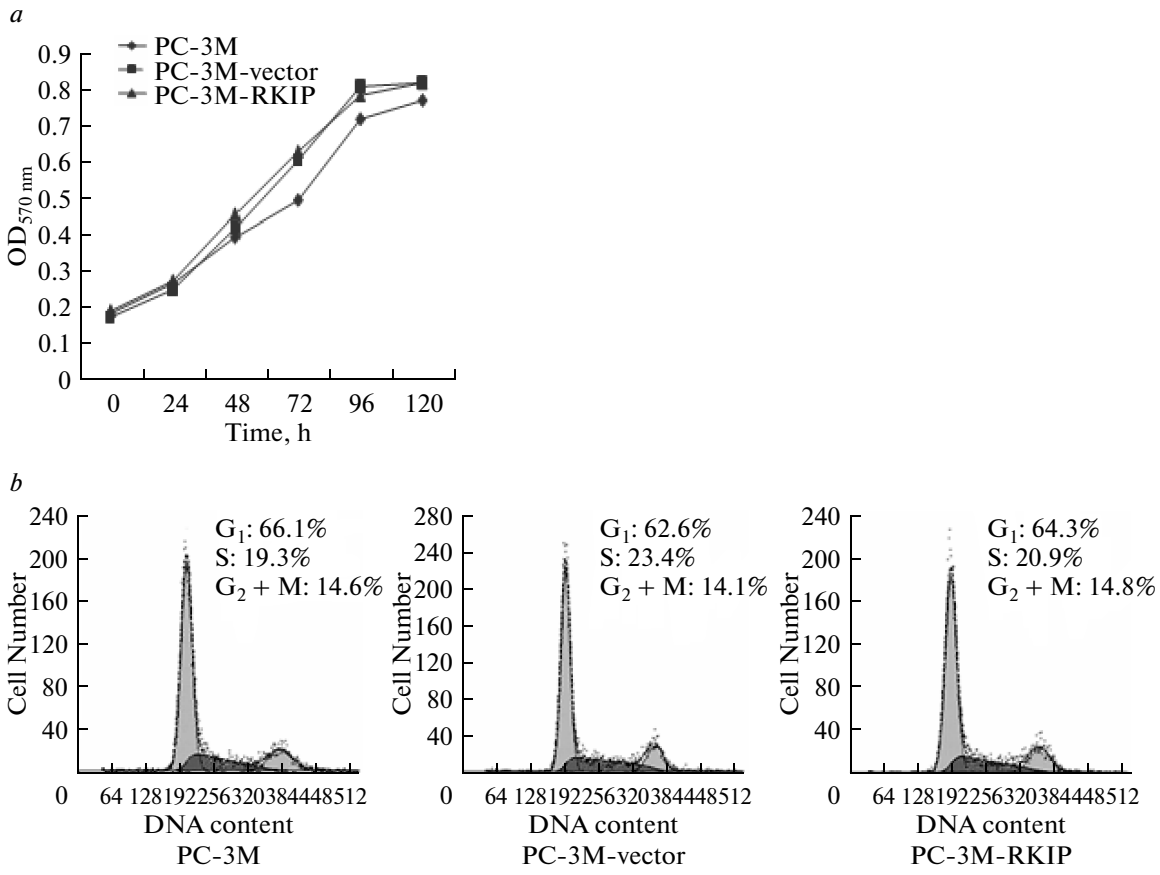

Fig. 2. The effect of RKIP on the proliferation of PC-3M cells. *a* – MTT assay; *b* – cell cycle analysis by flow cytometry.

firmed by RT-PCR and Western blotting analyses. As it can be seen (fig. 1*b*, *c*), RKIP expression was significantly higher in the cells transfected with a RKIP expressing vector as compared with those transfected with an empty vector.

To check the effect of RKIP on cell proliferation, we performed a MTT assay and a cell cycle assay to evaluate the proliferation of PC-3M ectopicaly expressing RKIP *in vitro*. The results indicated that RKIP did not inhibit the proliferation of PC-3M cells (fig. 2*a*, *b)*.

To evaluate the migration potential of PC-3M with ectopic expression of RKIP *in vitro*, wound healing and migration assays were performed. The result showed that PC-3M cells motility was suppressed by RKIP (fig. 3*a*–*c*). To evaluate the effect of RKIP on invasive ability of PC-3M cells, an *in vitro* invasion assay was carried out. The number of cells passing through the filter was markedly less in the experiment group with cells overexpressing RKIP than in the control ones (fig. 3*d*, *e*). These results suggested that ectopic expression of RKIP suppressed PC-3M cells migration and invasion *in vitro*.

RKIP regulates the expression and activity of metastasis related proteases

Since ectopic expression of RKIP could significantly suppress PC-3M cells migration and invasion *in vitro*, we further investigated the effect of ectopic expression of RKIP on the expression of genes that are involved in the migration and invasion of cancer cells. The mRNA levels of these genes were determined by RT-PCR. As shown in fig. 4*a*, ectopic expression of RKIP markedly inhibited the expression of MMP-2, MMP-9, uPA and cathepsin B at the mRNA level.

To determine whether suppression of PC-3M cell migration by ectopic expression of RKIP was associated with the inactivation of MMPs, conditioned medium was concentrated and analyzed by zymography with gelatin as a substrate. The results indicated that the proteolytic activity significantly decreased in the cells ectopicaly expressing RKIP (fig. 4*b*). Expression levels for uPA and cathepsin B proteins were determined by Western blotting. As shown in fig. 4*c*, ectopic expression of RKIP reduced the amounts of cathepsin B and uPA simultaneously.

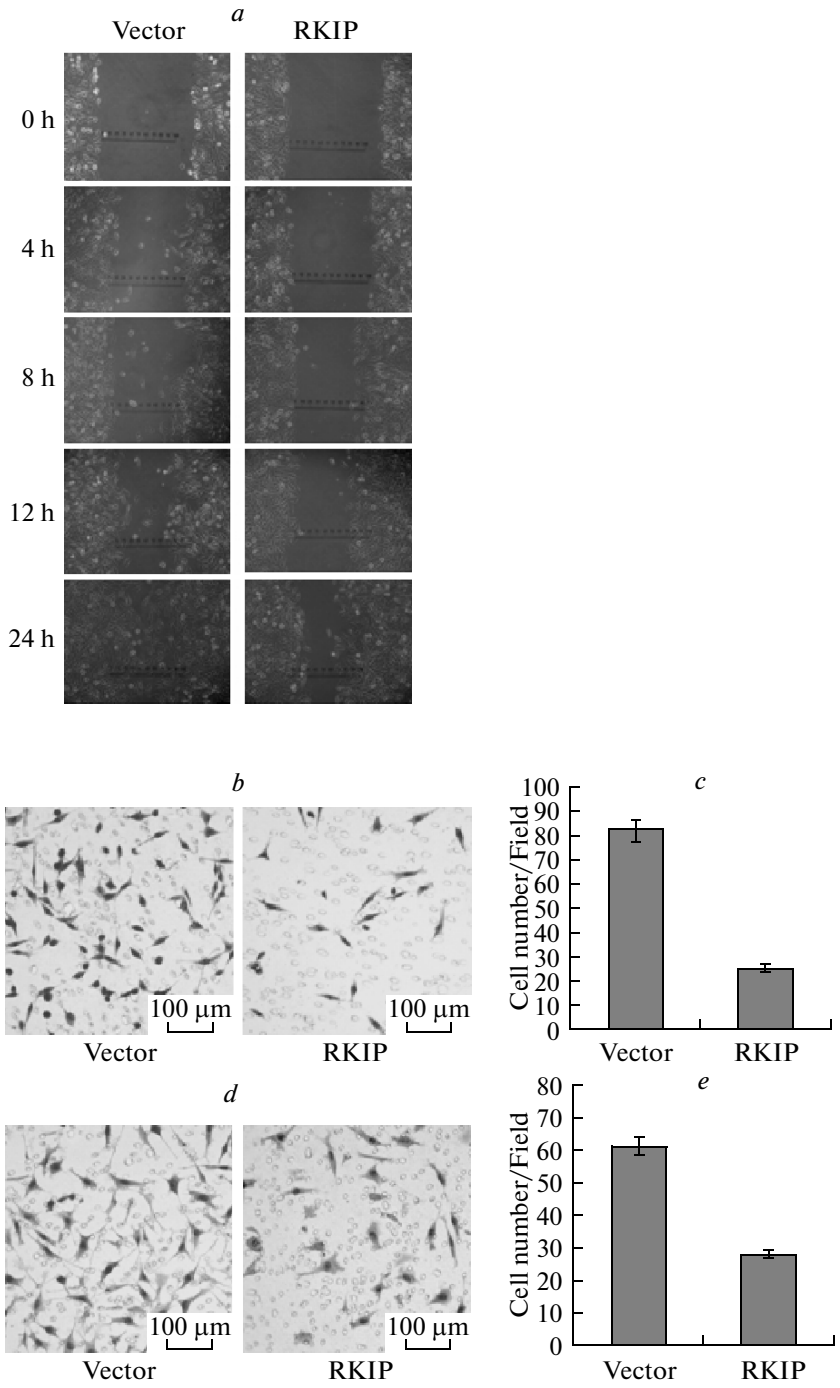

Fig. 3. RKIP inhibited the migration ability of PC-3M cells. *a* – "wound healing" migration assay (magnitude, ×100).The wound was created in a PC-3M cell culture as described in the Experimental section. Pictures of the wound closure were taken at 0 h, 4 h, 8 h, 12 h, 24 h as indicated and subjected to image anlysis. *b* –Boyden chamber migration assay. Images show the migration ability of PC-3M cells (magnitude, ×100). *c* – An average amount of cells was counted in five random fields.Tumor cell migration is markedly inhibited by ectopic expression of RKIP ($p < 0.01$ using the student's t-test) in comparison with the control. *d* – Matrigel invasion assay using PC-3M cells in culture as described in the Experimental. Images show the invasion ability of PC-3M cells (magnitude, ×100). *e* – An average amount of cells was counted in five random fields. Ectopic expression of RKIP resulted in a significant reduction in cell invasion ability ($p < 0.01$ using the student's t-test) in comparison with the vehicle treatment group.

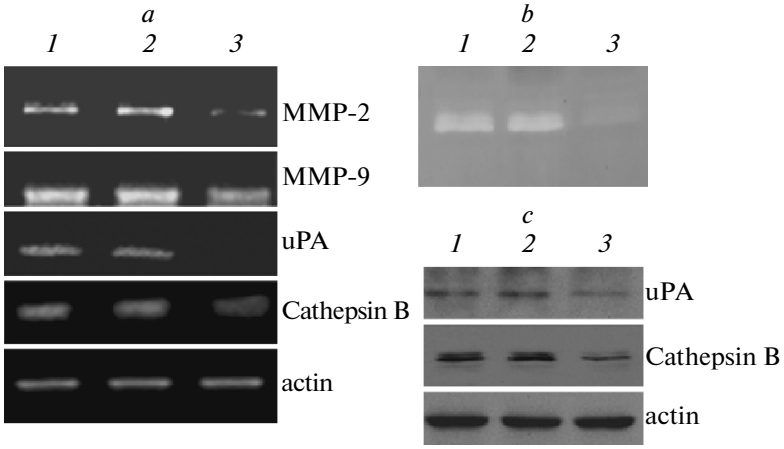

Fig. 4. Expression and activity of the metastasis related genes. *a* – Effect of ectopic expression of RKIP on the mRNA level of MMPs, cathepsin B and uPA. Equal amounts of RNAs were reverse transcribed to generate corresponding cDNAs for PCR analysis of the mRNA expression levels in PC-3M cells. *b* – gelatin zymography of the activity of MMPs in PC-3M. *c* – The effect of ectopic expression of RKIP on the expression of cathepsin B and uPA: the cell lysate proteins were separated by SDS-PAGE and analyzed with antibodies against cathespin B and uPA.

The effect of RKIP on expression of E-cadherin, NF-κB and Snail

The NF-κB (*full name, pls*) signal pathway is involved in regulating the expression of MMP-2 and MMP-9. RKIP inhibited the NF-κB activity through direct interactions with NF-κB-inducing kinase (NIK) and transforming growth factor-activated kinase 1(TAK1) [12]. Snail was shown to suppress RKIP transcription and expression [13]. Overexpression of Snail in cancer cells is responsible, in part, for the induction of epithelial-to-mesenchymal transition (EMT) through the down-regulation of E-cadherin [14], and Snail is transcriptionally regulated by NF-κB [15, 16]. Hence, we assessed the effect of RKIP on the expression of NF-κB and Snail in PC-3M cells. Western blotting analysis showed that RKIP induced an apparent decrease in the level of p65 and an increase in the amount of E-cadherin. But the ectopic expression of RKIP does not affect the level of Snail (fig. 5).

DISCUSSION

Raf kinase inhibitory protein (RKIP), an inhibitor of key signal transduction cascades in mammalian cells, regulates growth and differentiation. More and more data prove that RKIP is one of the promising metastasis suppressors. But the underlying molecular mechanisms are still unclear.

Metastasis is a complicated procedure involving lots of steps. Tumor cells must detach from the primary tumor, enter the circulatory system, and proliferate in the parenchyma of distant organs. The first step is to disrupt local cell-cell interactions through proteolytic degradation of the extracellular matrix (ECM) by proteases. Loss of cadherin based cell–cell adhesion and integrin based ECM adhesion plays an important role in cell migration and invasion [17]. A major component of intercellular adhesion in epithelia is the E-cadherin/catenin complex [18]. E-cadherin regulates the lamellipodia activity and cell migration directionality [19]. The loss of the E-cadherin gene expression can

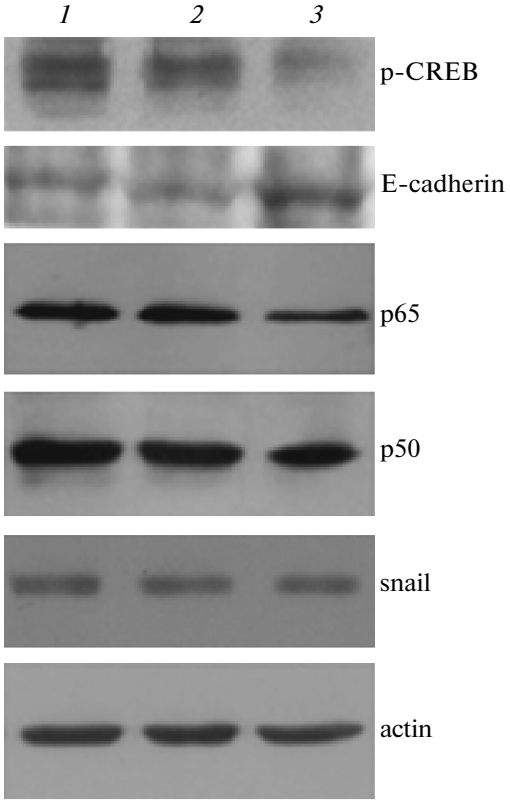

Fig. 5. The effect of RKIP on the expression of phorspho-rylated CREB, NF-κB, E-cadherin and Snail. Cell lysate proteins were separated by SDS-PAAG and analyzed with antibodies against E-cadherin, p50, p65, Snail and β-actin.

cause the dysfunction of the cell-cell junction, which triggers tumor metastasis [20–22]. As it has been shown in this study, RKIP induces an apparent increase in the protein level of E-cadherin. This result suggests that RKIP can promote intercellular adhesion and inhibit the migration of cancer cells.

ECM remodeling is controlled by proteases, including serine proteases (the plasminogen activators uPA and tPA), matrix metalloproteinases (MMPs), and cysteine proteases (cathepsins B, D, L and H) [23]. Especially, the activity of MMP-2 and MMP-9 is often found to be elevated in tumor tissues and malignant cancer cells. In this study it has been found that RKIP inhibits the expression of cathepsin B and uPA, and down-regulates gelatinase B. The results suggest that besides up-regulating of E-cadherin, RKIP also affects the ECM remodeling by down-regulating the proteases (fig. 4).

Snail was reported to be a repressor of RKIP transcription in metastatic prostate cancer cells [13]. Here, we analyzed whether ectopic expression of RKIP had a feed back effect on the expression of Snail and showed that ectopic expression of RKIP does not affect the level of Snail. Given that Snail is transcriptionally regulated by NF-κB, we also analyzed whether RKIP down-regulated the NF-κB signaling pathway and demonstrated that RKIP reduces amounts of p65 and phospho-CREB. There is a possible explanation that Snail is regulated by multiple signaling pathways and RKIP is an inhibitor of the key signal transduction cascades in mammalian cells. The effect caused by the ectopic expression of RKIP is balanced by the crosstalk between the signal pathways; so that the level of Snail is not affected by the expression of RKIP.

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