**RhoC Promotes Metastasis via Activation of the Pyk2 Pathway in Prostate Cancer**

Megumi Iiizumi,¹ Sucharita Bandyopadhyay,² Sudha K. Pai,¹ Misako Watabe,¹ Shigeru Hirot,³ Sadahiro Hosobe,¹ Taisei Tsukada,¹ Kunio Miura,¹ Ken Saito,¹ Eiji Furuta,¹ Wen Liu,¹ Fei Xing,¹ Hiroshi Okuda,¹ Aya Kobayashi,¹ and Kouosuke Watabe¹

¹Department of Medical Microbiology, Immunology, and Cell Biology, Southern Illinois University School of Medicine, Springfield, Illinois; ²Department of Developmental Biology, Stanford University School of Medicine, Stanford, California; and ³Akita Red Cross Hospital, Akita City, Japan

**Abstract**

RhoC is a member of the Ras-homologous family of genes which have been implicated in tumorigenesis and tumor progression. However, the exact role of RhoC is controversial and is yet to be clarified. We have examined the effect of RhoC on prostate tumor cells and found that RhoC had no effect on cell proliferation in vitro or on tumor growth in mice. However, RhoC significantly enhanced the metastatic ability of the tumor cells in these animals, suggesting that RhoC affects only the metastasis but not the growth of prostate tumor cells. The results of our immunohistochemical analyses on tumor specimens from 63 patients with prostate cancer indicate that RhoC expression had no significant correlation with Gleason grade. However, the expression of RhoC showed significant positive correlation with both lymph node and distant metastasis, and it was inversely correlated with patient survival. We also found that RhoC significantly augmented the invasion and motility of prostate tumor cells by activating matrix metalloproteinases 2 and 9 (MMP2 and MMP9) in vitro. The results of our antibody array analysis for signal molecules revealed that RhoC significantly activated kinases including mitogen-activated protein kinase (MAPK), focal adhesion kinase (FAK), Akt, and Pyk2. Inhibition of Pyk2 kinase blocked the RhoC-dependent activation of FAK, MAPK, and Akt, followed by the suppression of MMP2 and MMP9. Inhibitors of both MAPK and Akt also significantly blocked the activities of these MMPs. Therefore, our results indicate that RhoC promotes tumor metastasis in prostate cancer by sequential activation of Pyk2, FAK, MAPK, and Akt followed by the up-regulation of MMP2 and MMP9, which results in the stimulation of invasiveness of tumor cells. [Cancer Res 2008;68(18):7613–20]

**Introduction**

The family of Ras homologous (Rho) genes, which plays a central role in cell proliferation and motility, has been implicated in tumorigenesis as well as metastatic progression (1). The Rho subfamily includes RhoA, RhoB, and RhoC and they share 85% amino acid sequence identity (2). Despite this similarity, each protein has different affinities with various downstream effectors and shows different subcellular localizations, suggesting that they have distinct roles in normal cellular function as well as in tumor pathogenesis (3). RhoA seems to be involved in the regulation of actomyosin contractility, and the overexpression of RhoA has been shown to promote the invasiveness of tumor cells (2, 4–6). On the other hand, RhoB plays a role in controlling cytokine trafficking as well as in apoptosis induced by DNA-damaging agents and has been suggested to act as a suppressor of tumor progression (7, 8).

Recently, RhoC has been shown to be up-regulated in various types of cancer including inflammatory breast cancer (9), hepatocellular carcinoma (10), and non–small cell lung cancer (11). However, the exact role of RhoC in tumorigenesis and tumor progression has remained controversial and needs further clarification. Pille and colleagues previously found that blocking RhoC expression by short interfering RNA significantly inhibited cell proliferation of breast tumor cells in vitro as well as tumor growth in an animal model (12). More recently, Faried and colleagues also reported that ectopic expression of RhoC in esophageal carcinoma cells significantly enhanced the growth of tumors in nude mice. These results suggest that RhoC plays a critical role in cell proliferation and tumor growth both in vitro and in vivo (13). On the contrary, Ikoma and colleagues reported that ectopic expression of RhoC using retroviral vectors in Lewis lung carcinoma cells showed no significant difference in primary tumor growth in mice. However, the rate of lymph node metastasis was significantly enhanced in these animals (14). In agreement with these results, Hakem and colleagues recently constructed a RhoC knockout mouse and found that loss of RhoC does not affect tumorigenesis but significantly decreased metastasis in this mouse, suggesting that RhoC is involved only in metastasis but not in tumor cell proliferation (15). These apparent contradictory results by different groups may be due to the difference in the systems used or it may be due to the dependency of RhoC on cellular context. Therefore, it is critical to take a more systematic approach of testing the gene both in vitro and in vivo and to validate the outcome results in a clinical setting for each organ or tissue type in order to further clarify the role of RhoC in tumor progression. In this study, we found that RhoC promotes tumor metastasis but not tumor growth by sequential activation of Pyk2, focal adhesion kinase (FAK), mitogen-activated protein kinase (MAPK), and Akt followed by up-regulation of matrix metalloproteinases 2 and 9 (MMP2 and MMP9) in prostate tumor cells, and that the expression of RhoC serves as a marker to predict metastatic status and survival of patients with prostate cancer.

Note: Supplementary data for this article are available at Cancer Research Online (http://cancerres.aacrjournals.org/). Requests for reprints: Kounosuke Watabe, Department of Medical Microbiology, Immunology and Cell Biology, Southern Illinois University School of Medicine, 825 North Rutledge Street, Springfield, IL 62702, Phone: 217-545-3969; Fax: 217-545-3227; E-mail: kwatabe@siu.edu.

©2008 American Association for Cancer Research. doi:10.1158/0008-5472.CAN-07-6700

Materials and Methods

Cell culture and reagents. Human prostate cancer cell line PC3 was obtained from American Type Culture Collection, and human prostate cancer cell line PC3MM was kindly provided by Dr. I.J. Friedler (The University of Texas M. D. Anderson Cancer Center, Houston, TX). The PC3MM/tet cell line was previously established as a derivative of PC3MM and contains the tetacycline-inducible suppressor. Rat prostate cancer cell line AT2.1 was a gift from Dr. C.W. Rinker-Schaeffer (University of Chicago, Chicago, IL). All cell lines were cultured in RPMI 1640 supplemented with 10% fetal bovine serum, streptomycin (100 µg/mL), penicillin (100 units/mL), and 250 µmol/L of dexamethasone at 37°C in a 5% CO2 atmosphere. The phosphoinositide-3-kinase (PI3K)/Akt inhibitor (LY294002) and the MAPK inhibitor (PD98059) were purchased from Sigma Co. and Calbiochem, respectively. FAK inhibitor (TAE226) was previously described and kindly provided by Dr. Honda (Novartis Pharma AG, Basel, Switzerland; ref. 16).

Construction of expression vectors. To generate a RhoC expression vector, CDNA of the RhoC gene was isolated by PCR amplification from a human cDNA library using a forward primer containing a Flag-tagged Kozak sequence and a reverse primer (5'-TCCAATAGGTGATGTTGTGGT-3'). Products were transferred into the mammalian expression vector pcDNA3 (Invitrogen). To construct a tetacycline-inducible RhoC expression plasmid, the fragment of the RhoC gene in pcDNA3 was subcloned into pcDNA3/T5 (Invitrogen) at the BamHI/XhoI site. The RhoC expression plasmids or the vector alone were transfected into the AT2.1, PC3MM, and PC3MM/tet cells using LipofectAMINE (Invitrogen). To establish stable clones, transfected cells were treated with G418 or hygromycin, and drug-resistant colonies were selected following testing RhoC expression by Western blot.

Short hairpin RNA. Five individual short hairpin RNAs (shRNA) against the Pyk2 gene were purchased from Open Biosystems. shRNA with a scrambled sequence was purchased from Addgene and used as a negative control. The shRNAs were transfected into the prostate cancer cells using LipofectAMINE (Invitrogen) according to the manufacturer's protocol, and the culture was further incubated for 48 h before harvesting the cells for assays.

Western blot analysis. Cells were collected and dissolved in loading dye solution (125 mmol/L Tris-HCl, 4% SDS, 20% glycerol, 10% 2-mercaptoethanol, and 0.04% bromophenol blue), boiled for 5 min and subjected to 10% SDS-PAGE. Proteins were transferred to nitrocellulose membranes that were then treated with antibodies against anti-Flag (Sigma-Aldrich), anti-β-tubulin (Upstate Biotechnology), anti-phospho-Pyk2 (Ty-579/582; Sigma-Aldrich), anti-Pyk2 (Cell Signaling Technology), anti-phospho-Akt (Ser473; Cell Signaling Technology), anti-Akt (Cell Signaling Technology), anti-phospho-FAK (Ty-977; Sigma-Aldrich), anti-FAK (Cell Signaling Technology), or anti-phospho-MAPK (Thr183; Sigma-Aldrich) or anti-MAPK (Cell Signaling Technology). The membranes were then incubated with horseradish peroxidase–conjugated secondary antibodies and visualized by the enhanced chemiluminescence plus system (Amersham Life Sciences).

Cell growth assay. Cell lines expressing or not expressing the RhoC gene were cultured in the RPMI 1650 medium. At each time point, cells were collected and dissolved in loading dye solution (125 mmol/L Tris-HCl, 4% SDS, 20% glycerol, 10% 2-mercaptoethanol, and 0.04% bromophenol blue), boiled for 5 min and subjected to 10% SDS-PAGE. Proteins were transferred to nitrocellulose membranes that were then treated with antibodies against anti-Flag (Sigma-Aldrich), anti-β-tubulin (Upstate Biotechnology), anti-phospho-Pyk2 (Ty-579/582; Sigma-Aldrich), anti-Pyk2 (Cell Signaling Technology), anti-phospho-Akt (Ser473; Cell Signaling Technology), anti-Akt (Cell Signaling Technology), anti-phospho-FAK (Ty-977; Sigma-Aldrich), anti-FAK (Cell Signaling Technology), or anti-phospho-MAPK (Thr183; Sigma-Aldrich) or anti-MAPK (Cell Signaling Technology). The membranes were then incubated with horseradish peroxidase–conjugated secondary antibodies and visualized by the enhanced chemiluminescence plus system (Amersham Life Sciences).

Immunohistochemical analysis. Formaldehyde-fixed and paraffin embedded tissue specimens from 63 patients with prostate cancer were obtained from surgical pathology archives of the Akita Red Cross Hospital (Akita, Japan). Four-micron-thick sections were cut from the paraffin blocks of prostate tumors and mounted on charged glass slides. The sections were deparaffinized and rehydrated, and antigen retrieval was done by heating the slide in 25 mmol/L of sodium citrate buffer (pH 9.0) at 80°C for 30 min. The slides were incubated overnight at 4°C with anti-RhoC antibody (Santa Cruz Biotechnology) or anti–phospho-Akt (Ser473; Cell Signaling Technology). The sections were then incubated with the horseradish peroxidase–conjugated anti-goat secondary antibody, and 3,3'-diaminobenzidine substrate chromogen solution (Envision Plus kit; DAKO, Corp.) was applied followed by counterstaining with hematoxylin. Immunohistochemical staining conditions with other antibodies (NDRG1, AR, and PTEN) were described previously (17). Results of the immunohistochemistry for RhoC were judged by two independent persons (M. Iizumi and K. Watabe) based on the intensity of staining combined with the percentage of cells with positive staining.

In vitro motility and invasion assay. For the motility assay, 1 × 105 cells were added to the cell culture inserts with microporous membrane without any extracellular matrix coating (Becton Dickinson) and RPMI medium containing 20% fetal bovine serum was added to the bottom chamber. The cells were then incubated for 24 h at 37°C, and the upper chamber was removed. The cells on the bottom of the upper chambers were stained with tetrazolium dye, and the number of cells was counted under a microscope. For the in vitro invasion assay, the working method was similar to that described above, except that the inserts of the chambers to which the cells were seeded were coated with Matrigel (Becton Dickinson).

Wound-healing migration assay. Cells were seeded in a 10-cm dish and cultured to confluency. The cell monolayer was then scraped in the form of a cross with a plastic pipette tip. Three “wounded” areas were marked for orientation and photographed by a phase contrast microscope before and after 24 h of incubation.

Real-time reverse transcription-PCR. Forty-eight hours after transfection of appropriate plasmid DNA to the cells or 48 h after induction by tetraacycline, total RNA was isolated from the cells and reverse transcribed using random hexamer and MuLV reverse transcriptase (Applied Biosystems). The cDNA was then amplified with a pair of forward and reverse primers for RhoC (5'-TATAAGAGACATTAGGACAG and 5'-ATCCAGA- GAATGGGACAGC) or for the housekeeping genes, 18S rRNA (5'-TGTGGTGCTCGTGCTGGAAG and GACACGTGAAGCTTGCTGCTGCTG). PCR products were then visualized on a 1% agarose gel.

Gelatin zymograph assay. For zymography assay, cells (2.5 × 105) were seeded in 12-well plates and incubated for 48 h. Supernatants were collected and mixed with sample buffer followed by electrophoresis on a 10% SDS-polyacrylamide gel containing 5 mg/mL of gelatin. The gel was washed with 2.5% Triton X solution for 2 h and further incubated in the reaction buffer (50 mmol/L Tris-HCl, 5 mmol/L CaCl2, 1 µmol/L ZnCl2, and 1% Triton X-100) for an additional 18 h at room temperature. The gel was then stained with 0.5% Coomassie blue for 9 h and subsequently immersed with destaining buffer (30% methanol, 10% acetic acid) for 12 h. The stained gel was photographed and the intensity of each band was calculated digitally.

Antibody microarray. Antibody microarray was performed using a Panorama Antibody Microarray-Cell Signaling kit (Sigma-Aldrich) according to the manufacturer's instructions. Briefly, 1.5 × 105 cells were seeded in T-75 flasks and incubated for 48 h in the medium with or without tetracycline. Cells were collected and protein samples were prepared according to the manufacturer's protocol. These protein samples were labeled with Cy3 or Cy5 (Amersham Biosciences, UK) and subjected to antibody microarray (Sigma-Aldrich) analysis. The array slides were
scanned by GenePix Personal 4100A scanner (Molecular Devices) and the data was analyzed by GenePix Pro 5.0 (Molecular Devices).

Statistical analysis. For in vitro experiments and animal studies, t test or one-way ANOVA was used to calculate the P values. The association between RhoC and other clinical markers was calculated by χ² test. The Kaplan-Meier method was used to calculate the overall survival rate, and prognostic significance was evaluated by the log-rank test. Univariate and multivariate analyses for the prognostic value of RhoC was performed by the Cox proportional hazard-regression model. For all of the statistical tests, the significance was defined as P < 0.05. SPSS software was used in all cases.

Results

RhoC promotes tumor metastasis, but not cell growth.

To understand the role of RhoC in prostate cancer, we first established permanent cell lines expressing RhoC using the rat prostate carcinoma cell line, AT2.1, which has a poor metastatic potential (18). These cell lines expressing RhoC (clone no. 6 and no. 10) and a clone containing only the vector as well as the parental cell line, AT2.1, were individually injected s.c. into SCID mice. The mice were monitored for the formation and the growth rate of tumors and then sacrificed 3 weeks after the inoculation of the cells. As shown in Fig. 1A, all of the clones and the parental cells formed primary tumors in the animals with similar growth rates during the 3-week period, suggesting that RhoC does not have an effect on tumorigenesis or tumor growth. On the other hand, the clones stably expressing RhoC showed a significantly higher incidence of lung metastases compared with the parental cell line and the vector-only clones (Fig. 1B). These results strongly suggest that RhoC can promote the metastatic process of prostate cancer cells without affecting tumorigenicity in vivo. We also examined the effect of RhoC on the growth of these cells in vitro. The results of a

colorimetric assay after 72 h indicate that there was no significant difference in the growth rate between the cells with and without RhoC (Supplementary Fig. S1A). We then examined the rate of DNA synthesis of the cells with and without the expression of RhoC and found that there was no significant difference between these cells (Supplementary Fig. S1B). Furthermore, we established a human prostate cell line, PC3MM/tet/RhoC, which contains the tetracycline-inducible RhoC gene, as well as PC3 cell lines that did or did not ectopically express RhoC. We then examined the rate of cell growth and DNA synthesis of these cells. Again, we found that RhoC did not affect the rate of proliferation of the cells (Supplementary Fig. S1A and B), which further supports our notion that RhoC has no apparent role in the growth of prostate cancer cells, although it significantly promotes tumor metastasis.

RhoC expression is significantly increased with the advancement of human prostate cancer.

To further corroborate our results in a clinical setting, we examined the status of RhoC expression and its relationship with different clinicopathologic factors in prostate cancer by immunohistochemical analysis of 63 prostate tumor specimens. They were randomly selected from surgical pathology archives dating from 1988 to 2001. As shown in Fig. 2A and B, the expression of RhoC was found to be strongly elevated in high-grade tumors, particularly in specimens from patients with metastatic disease, compared with normal prostatic tissue or low-grade tumors. The results of our statistical analyses indicate that RhoC is strongly expressed in tumors with higher Gleason grade, although the correlations are not statistically significant (Fig. 2B). Importantly, the RhoC expression showed significant positive correlation with the metastases status of the patients (P = 0.028). It was also noted that RhoC expression showed a significant inverse correlation to that of NDRG1 (P = 0.02), which

Figure 1. RhoC promotes tumor metastasis without affecting the primary tumor growth in vivo.

The RhoC expression plasmid was introduced into a low-metastasis rat prostate cell line, AT2.1, and clones (no. 6 and no. 10) that constitutively express RhoC were established. As a control, the original vector was also cloned into AT2.1. These clones, as well as the parental line, were injected s.c. into SCID mice as described previously. The volume of the primary tumor for each clone at the indicated time was measured using the equation: volume = (width + length) / 2 × W × L × 0.5236 (A). Inset, results of a Western blot of RhoC expression for each clone. Mice were sacrificed 3 wk after the inoculation of the cells, and metastatic lesions on the lungs were counted macroscopically (B). \( P < 0.05 \), statistically significant difference.

<table>
<thead>
<tr>
<th>Cell line</th>
<th>RhoC +</th>
<th>Tuor incidence +</th>
<th>In vivo doubling time</th>
<th>Lung metastasis (mean ± S.E.) +</th>
<th>P</th>
</tr>
</thead>
<tbody>
<tr>
<td>AT2.1</td>
<td>–</td>
<td>7/7</td>
<td>4.1 ± 0.5</td>
<td>3.1 ± 1.3</td>
<td></td>
</tr>
<tr>
<td>Vector only</td>
<td>–</td>
<td>4/4</td>
<td>3.9 ± 0.3</td>
<td>2.0 ± 1.1</td>
<td></td>
</tr>
<tr>
<td>RhoC no. 6</td>
<td>+</td>
<td>8/8</td>
<td>3.9 ± 1.1</td>
<td>10.9 ± 3.5</td>
<td>0.04$</td>
</tr>
<tr>
<td>RhoC no. 10</td>
<td>+</td>
<td>5/5</td>
<td>3.8 ± 0.7</td>
<td>9.4 ± 2.4</td>
<td>0.03$</td>
</tr>
</tbody>
</table>

* RhoC expression was examined by Western blot.
† Number of tumor-bearing SCID mice / no. of tumor-inoculated SCID mice.
‡ Number of metastatic lesions on lungs per SCID mouse.
$ P < 0.05.
has recently been shown to be a tumor metastases suppressor in prostate cancer (19). These results suggest that the expression of RhoC is up-regulated at a relatively late stage and is directly involved in metastatic progression of prostate cancer, which is in good agreement with our in vivo data. Furthermore, the results of our survival analyses on 50 patients with prostate cancer over a period of 5 years indicates that patients with positive expression of RhoC had significantly worse overall survival rate than the patients with a reduced expression of the gene ($P = 0.018$, log-rank test; Fig. 3). The results of univariate Cox regression analysis revealed that the death risk of patients with increased RhoC expression was 4.8 times higher than the risk of patients with RhoC negativity. However, when we performed a multivariate analysis for RhoC, Gleason score, and metastasis, only the metastasis status gave a significant value ($P = 0.015$) and other two factors were excluded. The fact that multivariate analyses of these three factors excluded RhoC status indicates that the profiles of the RhoC expression and metastasis status of patients significantly overlaps and that each factor has enough "power" for predicting patient outcome. In fact, when we did a multivariate analysis for a combination of RhoC status and Gleason score, which is the most widely used pathologic marker for prostate cancer, RhoC status turned out to be a better predicting marker than Gleason score ($P = 0.037$ and $P = 0.237$ for RhoC and Gleason score status, respectively). Although RhoC expression did not significantly and independently predict survival compared with metastasis, increased RhoC correlates with aggressive disease which could account for increased metastatic disease.

RhoC promotes invasiveness and motility of prostate cancer cells in vitro. To understand how RhoC contributes to the progression of prostate cancer, we ectopically expressed the RhoC gene in the human prostate cancer cell line, PC3, followed by examining the invasiveness and migration of the cells in vitro. We found that the expression of RhoC significantly enhanced both cell
invasiveness and migration ($P = 0.03$ and $0.004$, respectively; Fig. 4A), which is in good agreement with the previous results of Yao and colleagues (20). The effect of RhoC on cell motility was also examined by the "wound healing" assay. As shown in Fig. 4B, cells with ectopically expressing RhoC showed a much higher rate of motility compared with the cells with an empty vector transfectant. These results strongly suggest that RhoC promotes metastasis by enhancing the invasiveness and/or motility of tumor cells. Because the invasive ability of tumor cells is known to often be correlated with their production of secretory proteases (21), we examined the expression of MMP2 and MMP9 in the cells that overexpressed RhoC. As shown in Fig. 4C, quantitative reverse transcription-PCR (qRT-PCR) analysis for the cell overexpressing RhoC significantly augmented the level of the expression of the MMP2 and MMP9 genes ($P = 0.049$ and $0.02$, respectively). These results were further validated by gelatin zymography and Western blot analyses as shown in Fig. 4D. Therefore, our results indicate that the invasiveness of tumor cells induced by RhoC is, at least in part, due to the overexpression of MMP2 and MMP9.

RhoC activates MMP through the Pyk2 signal pathway. To gain further insight into the signaling pathways by which RhoC promotes the invasive phenotype, we prepared cell lysates from PC3MM/tet/RhoC with or without induction of the RhoC gene by tetracycline. The lysates were labeled with Cy3 and Cy5 and analyzed on an antibody microarray which contained 224 antibodies for various key molecules of cell signaling and cell cycle, and the results of ratios were rank-ordered. As shown in Fig. 5A (left), ectopic expression of RhoC significantly phosphorylated a series of protein kinases including MAPK, FAK, Akt, and Pyk2. The result of the array analysis was also confirmed by Western blot using the antibodies specific to phosphorylated proteins as well as the antibodies to the total proteins for each signal molecule (Fig. 5A, right; Supplementary Fig. S2A). These results suggest that RhoC can directly activate a cascade of signal pathways involving these key signal molecules that are closely related to cell motility and tumor progression.

Pyk2 is a tyrosine kinase and belongs to a member of the FAK subfamily which plays a critical role in cell migration and motility of various cell types (22, 23). Pyk2 is also known to be able to phosphorylate Akt (23). Therefore, we investigated the possibility

![Figure 3](image_url) Prognostic value of RhoC expression. Overall survival rate over a period of 5 y was calculated in 50 patients with prostate cancer in relation to the expression of the RhoC genes by Kaplan-Meier method. $P = 0.018$ was determined by a log-rank test. RhoC-positive (solid line) patients and patients with reduced expression (dotted line) of RhoC.

![Figure 4](image_url) RhoC promotes invasiveness and motility of prostate cancer cells in vitro. A, the RhoC expression plasmid (pcDNA3/RhoC) or the vector alone was transfected into the PC3 cell line. After 24 h, cells were collected and subjected to invasion (left) and migration (right) assays. *, $P < 0.05$, statistically significant difference. B, for the motility assay, the PC3 cells stably transfected with the RhoC expression plasmid or an empty vector were cultured to confluency. The monolayer was scratched by drawing lines and photographed under a microscope. After 24 h of incubation, they were photographed again. C, to test the effect of RhoC on MMP2 and MMP9, PC3 cells that have been stably transfected with the RhoC expression plasmid or an empty vector were cultured in 12-well plates. Cells were then collected and their total RNA was treated with DNase. The RNA was then subjected to qRT-PCR using specific primers for the RhoC, MMP2, and MMP9 genes. Results were presented as ratios of the expression level of each gene in RhoC-positive and RhoC-negative cells. *, $P < 0.05$, statistically significant difference. D, MMP2 and MMP9 activities in the conditioned medium from the PC3 cells with or without the RhoC expression plasmid as described in C were assayed by gelatin zymography. The image was photographed and the intensity of each band was digitally quantified. The expression of Flag-RhoC was confirmed by Western blot (top).
Figure 5. RhoC activates MMPs through the Pyk2/FAK pathway. A, for antibody array analysis, cell lysates were prepared from the PC3MM/tet cells containing the tetracycline-inducible RhoC gene with or without induction of RhoC. The proteins were labeled with Cy3 or Cy5 and subjected to antibody microarray (Sigma-Aldrich) analysis. The scanned data was analyzed by GenePix Pro 5.0 (Axon Instrument). The result of the antibody array data was confirmed by Western blot using phosphospecific antibodies to Pyk2, FAK, MAPK, and Akt as well as using antibodies to the total protein of each corresponding gene. B, PC3 cells stably transfected with the RhoC-expression plasmid or an empty vector were transfected with the expression plasmid of shRNA for Pyk2 or a scrambled sequence. After 48 h, cells were collected and subjected to Western blot analysis using phospho-specific antibodies (left). To examine the effect of Pyk2 and MAPK on the MMP expression, the same set of cells were treated with or without the MAPK inhibitor, PD98059 (100 μmol/L) for 48 h. RNA was extracted from each sample (in triplicate) and subjected to qRT-PCR using specific primers for MMP2 and MMP9 (right). C, the effect of Akt phosphorylation on MMP expression was examined. Cells with or without expression of RhoC were treated with or without PI3K/Akt inhibitor, Ly294002 (100 nmol/L), for 48 h. The cells were then collected and RNA was extracted followed by qRT-PCR analysis for MMP2 and MMP9 expression (left). The conditioned culture mediums of the same set of samples were subjected to zymography assay for MMP2 and MMP9 (right). The image was photographed and the intensity of each band was digitally quantified. D, to examine the clinical status of RhoC and p-Akt expression, 27 samples from patients with prostate cancer were analyzed by immunohistochemistry using antibodies to RhoC and p-Akt. The result was analyzed by χ² test. E, PC3 cells with or without RhoC expression were treated with shPyk2 or the FAK-specific inhibitor, TAE226, for 48 h. The cells were then assayed for their invasiveness by using a Matrigel invasion assay as described in Materials and Methods.
that Pyk2 is an immediate effector of the RhoC signal and that it controls the downstream pathways. PC3/RhoC cells were transfected with the expression vector of shRNA targeted to Pyk2. After 48 h of incubation, cell lysates were prepared and subjected to Western blot analysis using antibodies to RhoC, p-FAK, p-MAPK, and p-Akt. As shown in Fig. 5B (left) and Supplementary Fig. S2B, induction of RhoC strongly phosphorylated FAK, MAPK, and Akt, and this RhoC-dependent phosphorylation of these molecules was strongly blocked by the addition of shRNA to the Pyk2 gene, suggesting that RhoC first activates Pyk2, which then phosphorylates FAK, MAPK, and Akt. We then examined whether MMP2 and MMP9 are indeed activated by Pyk2 and MAPK in a RhoC-dependent manner. RNA was prepared from PC3/RhoC cells that were cultured in the presence or absence of shRNA for Pyk2 and the MAPK inhibitor, PD98059. RNAs were then examined for the expression of MMP2 and MMP9 by qRT-PCR. As shown in Fig. 5B (right) and Supplementary Fig. S2C (left), RhoC-dependent activation of both MMP2 and MMP9 was significantly abrogated in the presence of shRNA for Pyk2 or the MAPK inhibitor, suggesting that the activation of MMP2 and MMP9 by RhoC is at least partly due to the phosphorylation of Pyk2 followed by the activation of MAPK. Because our results indicate that Akt is also phosphorylated at Ser273 by RhoC in a Pyk2-dependent fashion, we examined whether Akt is also involved in the activation of MMP2 and MMP9 in the RhoC signal pathway. As shown in Fig. 5C (left) and Supplementary Fig. S2C (right), we found that the RhoC-dependent induction of MMP2 and MMP9 was indeed significantly blocked by PI3K/Akt inhibitor, Ly294002. This result was further confirmed by gelatin zymography analysis as shown in Fig. 5C (right). To further corroborate the in vitro results, we examined 27 clinical specimens from patients with prostate cancer by conducting immunohistochemistry using anti-RhoC and anti-phospho-Akt (Ser473) antibodies. As shown in Fig. 5D, we found that RhoC expression was significantly correlated with the expression of phospho-Akt in these tumor tissues. Therefore, these clinical data as well as the in vitro results strongly suggest that Akt is part of the downstream effectors of RhoC signals and plays an important role in RhoC-dependent activation of MMP2 and MMP9. To further validate the role of Pyk2 and FAK in the RhoC-induced signal, we treated the PC3 cells that do or do not express RhoC with shPyk2 or the FAK-specific inhibitor, TAE226, followed by measuring the invasiveness of these cells using the Matrigel invasion chamber assay. As shown in Fig. 5E, we found that inhibition of Pyk2 and FAK indeed significantly blocked the RhoC-induced invasiveness of the prostate tumor cells, which strongly suggests the functional involvement of Pyk2 and FAK in the RhoC signaling pathway.

Discussion

RhoC has been shown to be involved in various types of tumors (9–11). However, the exact role of RhoC in tumor progression and its underlying mechanism are unclear, and the previous results from different groups have presented an apparently contradictory picture of the function of this gene (12–15). In this study, we have integrated multiple approaches, both in vitro and in vivo, to clarify the functional role of RhoC in prostate cancer progression. The results of our animal experiments clearly indicate that RhoC plays a critical role in the metastatic progression of prostate tumor but it is not essential for tumor cell growth. The results of immunohistochemical analysis of human prostate cancer specimens also indicates that RhoC expression is significantly correlated with the metastatic status of the patients but not with Gleason grade, which strongly supports our notion that RhoC is implicated mainly in the metastatic process but not in tumorigenesis. Importantly, RhoC expression is inversely correlated with patient survival, suggesting that RhoC can serve as a prognostic marker as well as a potential therapeutic target for prostate cancer.

The molecular mechanism by which RhoC promotes tumor progression is an intriguing question. We have constructed a RhoC-inducible cell line and examined its protein expression profile using an antibody array to clarify the signal pathway. The results of the array analysis revealed that Pyk2, FAK, MAPK, and Akt were all phosphorylated upon induction of the RhoC expression, and the knockdown of Pyk2 resulted in significant reduction in phosphorylation of FAK, MAPK, and Akt, suggesting that Pyk2 is the upstream effector and plays a central role in the RhoC signal pathway. Pyk2 belongs to the subfamily of focal adhesion protein tyrosine kinases and it has been shown to be involved in cell migration, invasion, and proliferation (24–28). It was reported that in the in vitro model of transforming growth factor-β-induced epithelial to mesenchymal transition, Pyk2 was strongly phosphorylated at Tyr881 whereas during migration, Pyk2 was strongly phosphorylated at Tyr580 (22). It should be noted that, in our antibody array analyses, both of these sites were found to be phosphorylated (Fig. 5A). Pyk2 is capable of transducing signals via several known pathways, and one of the effectors is FAK which has been shown to be phosphorylated by Pyk2 at Tyr397, Tyr725, and Tyr635 (29). The results of our antibody array data also revealed that both of these sites were indeed phosphorylated upon induction of RhoC. These results suggest that RhoC activates FAK via phosphorylation of Pyk2. FAK is a focal-adhesion kinase and plays a critical role in cell migration and motility (30–32). The enhanced expression of FAK has been documented in a number of different types of human cancers (33–41). The phosphorylation of FAK is known to be linked to the activation of several downstream signals including ERK and JNK/MAPK as well as PI3K/Akt (42, 43). Furthermore, it was previously shown that the invasive ability of RhoC was significantly attenuated by a MAPK inhibitor in vitro (44). Notably, the results of our knockdown experiments using Pyk2-specific shRNA has shown that the RhoC-dependent phosphorylation of both ERK/MAPK and Akt was significantly blocked by knockdown of Pyk2, suggesting that MAPK and Akt are activated by RhoC via phosphorylation of Pyk2 and FAK.

We have shown that RhoC promotes metastasis by augmenting the motility and invasion of tumor cells (Figs. 4 and 5) via activation of MMP2 and MMP9, two key proteases for the invasion of tumor cells. It should be noted that the expression of both MMP2 and MMP9 was previously shown to be modulated by the activation of Akt and MAPK (45–47). We have indeed shown that inhibitors of both molecules significantly blocked the RhoC-dependent activation of MMP2 and MMP9. In this context, it should be noted that Ruth and colleagues have recently shown that RhoC promoted the invasion of human melanoma cells in a PI3K/Akt-dependent manner (48). Our results also indicate that Akt was significantly phosphorylated at Ser73 by RhoC, and that the phosphorylation of this serine residue has previously been found to be involved in the motility and invasiveness of tumor cells (45, 46, 49). The activation of Akt has also been shown to be clinically associated with aggressiveness and earlier recurrence of prostate cancer (50). Collectively, our results indicate that RhoC enhances the invasiveness and metastatic ability of tumor cells by
activating the Pyk2/FAK pathway followed by phosphorylation of Akt and MAPK, which in turn, activate MMP2 and MMP9. RhoC is considered to serve as an independent prognostic marker to predict patient outcome, and an intervention of the RhoC signal may be an effective therapeutic strategy for prostate cancer.

Disclosure of Potential Conflicts of Interest

No potential conflicts of interest were disclosed.

References

RhoC Promotes Metastasis via Activation of the Pyk2 Pathway in Prostate Cancer

Megumi Iiizumi, Sucharita Bandyopadhyay, Sudha K. Pai, et al.


Updated version
Access the most recent version of this article at:
http://cancerres.aacrjournals.org/content/68/18/7613

Supplementary Material
Access the most recent supplemental material at:
http://cancerres.aacrjournals.org/content/suppl/2008/09/08/68.18.7613.DC1

Cited articles
This article cites 50 articles, 20 of which you can access for free at:
http://cancerres.aacrjournals.org/content/68/18/7613.full.html#ref-list-1

Citing articles
This article has been cited by 10 HighWire-hosted articles. Access the articles at:
/content/68/18/7613.full.html#related-urls

E-mail alerts
Sign up to receive free email-alerts related to this article or journal.

Reprints and Subscriptions
To order reprints of this article or to subscribe to the journal, contact the AACR Publications Department at pubs@aacr.org.

Permissions
To request permission to re-use all or part of this article, contact the AACR Publications Department at permissions@aacr.org.