Hepatocellular carcinoma (HCC) is one of the most common malignancies in Asia and Africa, especially in China (1, 2). During the past decade, the long-term survival remains unsatisfactory because of a high incidence of recurrence and metastasis after the hepatic resection, with a 5-year actuarial recurrence rate of 75% to 100% reported in the literature (3). Thus, the inhibition of invasion and metastasis is of great importance in the HCC therapies.

Available information indicates that invasion and metastasis is to a large extent attributable to the ability of cell migration (4, 5). Rho-GTPases, members of Ras superfamily of small GTPases, shuttle between inactive GDP-bound and active GTP-bound form and exhibit intrinsic GTPase activities. Activation of the Rho protein leads to the assembly of the actin-myosin contractile filaments into focal adhesion complexes that lead to cell polarity and facilitate motility (6). Recently, RhoC has attracted interest because its increased expression has been linked to increased invasion in breast, melanoma, pancreatic, colon, bladder, hepatocellular, non-small cell lung carcinoma, and primary gastric tumor or cell lines (7–13). Our previous study revealed that RhoC was a prognostic marker with HCC and its expression was correlated with invasion and metastasis of HCC (14–16). Interestingly, a recent study using RhoC-deficient mice showed that loss of RhoC does not affect the development of palpable mammary adenocarcinoma but leads to a drastic inhibition of metastasis. The study also showed that inactivation of RhoC does not affect embryogenesis, normal cell functions, or immune response, implicating RhoC as an ideal therapeutic target of cancer cell metastasis (17).

Although a correlation between the overexpression of RhoC and the metastasis of HCC was observed previously, emerging data implicated RhoC as a potential therapeutic target for metastasis of human tumor (18, 19). However, the precise role of RhoC in HCC remains largely unknown. Does RhoC participate directly in the metastasis of HCC through increasing motility of HCC cells? Could it be an effective therapeutic target for metastasis of HCC? To address these questions, we used a stable retroviral small interfering RNA (siRNA) approach to selectively knockdown the expression of RhoC, which allowed...

Abstract

Purpose: Hepatocellular carcinoma (HCC) is one of the most deadly human cancers because of its high incidence of metastasis. Our previous work identified a strong correlation between increased expression of RhoC and HCC metastasis. Here, we investigate to define the role of RhoC in HCC metastasis. Furthermore, we sought to determine whether inhibition of the expression of RhoC might block the metastasis of HCC in vivo.

Experimental Design: A stable retroviral small interfering RNA approach was employed to selectively knockdown the expression of RhoC in vitro and in vivo. Invasion and migration assay, MTT and fluorescence-activated cell sorting analysis, Rho activity assay, and immunofluorescence staining were carried out to characterize RhoC in vitro. An anti-RhoC retroviral gene delivery BALB/c nude mice model was established to investigate whether knockdown of the expression of RhoC might inhibit the metastasis of HCC in vivo.

Results: We confirmed the correlation of RhoC expression and metastatic potentials of HCC cell lines. We also showed that suppression of RhoC expression resulted in inhibition of invasion and migration without an apparent effect on cell survival and proliferation in HCCLM3 cells. Furthermore, a similar effect of RhoC on autotaxin-induced invasion of HCCLM3 cells was also observed. Significantly, we successfully adopted an HCC metastatic mouse model that allowed us to show that knockdown of the RhoC expression resulted in inhibition of metastasis of HCC in vivo for the first time.

Conclusions: Our results show a critical role of RhoC in metastasis of HCC, implicating RhoC as a potential therapeutic target to block HCC metastasis.
Translational Relevance

HCC is one of the most deadly human cancers because of its high incidence of metastasis. Thus, the inhibition of invasion and metastasis is of great importance in the HCC therapies. In this study, we showed a critical role of RhoC in metastasis of HCC through enhancing invasion and migration of HCC cell. More importantly, we established successfully a HCC metastatic mouse model implanted s.c. with retrovirus packaging cells expressing RhoC siRNA and found that knockout RhoC expression might inhibit metastasis of HCC in vivo for the first time. These findings provide evidence indicating a critical and specific role of RhoC in metastasis of HCC. Furthermore, data from our present study imply a new cancer therapy that blocks HCC metastasis by specifically inhibiting RhoC.

Materials and Methods

Antibodies and reagents. Recombinant human autotaxin (ATX) was purchased from Phoenix Pharmaceuticals. Goat anti-RhoC antibody was purchased from Santa Cruz Biotechnology. Biotin anti-mouse phalloidin was purchased from Molecular Probes. Rho Activation Assay Kit was purchased from Upstate. pSUPER.retro.neo retroviral expression plasmid (Oligoengine) at 70% production of amphotropic virus, were transfected with relevant retroviral packaging cells, which express the 10A1 viral envelope for the production of amphotropic virus, were transfected with relevant pSUPER.retro.neo retroviral expression plasmid (Oligoengine) at 70% confluence with the use of Lipofectamine 2000 (Invitrogen) according to the manufacturer’s instruction. Clones expressing retrovirus at 1 × 10^5 colony-forming units/mL were selected for subsequent experiments. Multiple rounds of infection, which can increase the number of infected cells as well as the number of copies per cell, were done during the transfection of HCCLM3 cells. Briefly, medium containing retrovirus was added to the cells supplied with polybrene (8 μg/mL) for 12 h; after a rest for 12 h by replacing medium with fresh medium, retrovirus was added again. This doubt infection resulted in a 60% to 70% of transfection efficiency.

Generation of siRNA constructs. Three putative candidate sequences and one control sequences were determined by following the above protocol. The three putative candidate sequences and one control sequences were as follows: sequence 1 sense 5’-GATCCCCGAGCTTCCCTTATCCTGTCATGCAAGGATGAGGAGGAGGC-3’ and antisense 5’-AAGCTTGGACACTATGGTGGTCTTCCAATGAGTGGG-3’; sequence 2 sense 5’-GATCCCCGAGCCATAGTTCACCTGTCATGCAAGGATGAGGAGGAGG-3’ and antisense 5’-AAGCTTGGACACTATGGTGGTCTTCCAATGAGTGGG-3’; sequence 3 sense 5’-GATCCCCGAGCTTCCCTTATCCTGTCATGCAAGGATGAGGAGGAGG-3’ and antisense 5’-AAGCTTGGACACTATGGTGGTCTTCCAATGAGTGGG-3’; sequence 4 sense 5’-GATCCCCGAGCTTCCCTTATCCTGTCATGCAAGGATGAGGAGGAGG-3’ and antisense 5’-AAGCTTGGACACTATGGTGGTCTTCCAATGAGTGGG-3’; sequence 5 sense 5’-GATCCCCGAGCTTCCCTTATCCTGTCATGCAAGGATGAGGAGGAGG-3’ and antisense 5’-AAGCTTGGACACTATGGTGGTCTTCCAATGAGTGGG-3’; sequence 6 sense 5’-GATCCCCGAGCTTCCCTTATCCTGTCATGCAAGGATGAGGAGGAGG-3’ and antisense 5’-AAGCTTGGACACTATGGTGGTCTTCCAATGAGTGGG-3’; sequence 7 sense 5’-GATCCCCGAGCTTCCCTTATCCTGTCATGCAAGGATGAGGAGGAGG-3’ and antisense 5’-AAGCTTGGACACTATGGTGGTCTTCCAATGAGTGGG-3’.

RNA isolation and reverse transcription-PCR. The details of real-time quantitative reverse transcription-PCR have been described previously (14). The primers sequences were as follows: RhoA, 116 bp, sense 5’-GATCCCCGAGCTTCCCTTATCCTGTCATGCAAGGATGAGGAGGAGG-3’ and antisense 5’-TGGGAACTTTGTGCCTGTCC-3’; RhoB, 180 bp, sense 5’-GCGAACACTTTGGCCTTCTTCTCT-3’ and antisense 5’-ACAGCGTAAACTGTGGTCTGAAATG-3’; RhoC, 161 bp, sense 5’-AGGCCATGAAGAGGCAAGGATGCTTGTTGCAG-3’ and antisense 5’-CTCAACACGCTTCGCCAGC-3’; Rac1, 187 bp, sense 5’-CCCTGGATGTCCCTCGATCAATGG-3’ and antisense 5’-CATGGCTAGACCCTGCGTCTC-3’.

Cell culture and transfections. HCCLM3 and MHCC97-L cell lines, two HCC cell lines with differently metastatic potentials, were obtained from Liver Cancer Institute and Zhong Shan Hospital of Fudan University. HepG2 HCC cell line was provided by Cell Center of Xiangya Medical School. PT67 retroviral packaging cell line was purchased from BD Biosciences Clontech. These cell lines were cultured in high-glucose DMEM supplemented with 10% fetal bovine serum and incubated in 5% CO2 at 37°C. For transfection of packaging cells, PT67 retroviral packaging cells, which express the 10A1 viral envelope for the production of amphotropic virus, were transfected with relevant pSUPER.retro.neo retroviral expression plasmid (Oligoengine). Matrigel was purchased from Becton Dickinson.

Western blotting and Rho activity assays. Cells were lysed in a lysis buffer (Pierce). Extract equivalent to 100 μg total protein was separated on 12% polyacrylamide gels and transferred to polyvinylidene fluoride membrane (Invitrogen). The membrane was incubated with primary antibody (goat anti-RhoC polyclonal antibody, diluted at 1:500) followed by incubation with a 1:1,000 dilution of horseradish peroxidase-linked rabbit anti-goat antibody (Santa Cruz Biotechnology). Then, the membrane was washed and treated with Western blotting luminal reagent (Pierce) to visualize the bands. For Rho activity assays, cells were lysed in a lysis buffer supplied with cocktail protease inhibitors. Lysates (500 μg) were cleared at 13,000 × g for 10 min, and the supernatant was rotated for 30 min with 30 μg GST-RBD (GST fusion protein containing the Rho-binding domain of Rhotekin) bound to glutathione-Sepharose beads. Samples were washed in 50 mmol/L Tris (pH 7.4), 10 mmol/L MgCl2, 150 mmol/L NaCl, 1% Triton X-100, and protease inhibitors. GST-RBD pull-downs and lysates were then immunoblotted with an antibody specific for RhoC (C-16) from Santa Cruz Biotechnology (20).

Invasion and migration assay. For invasion assay, Transwell with an 8 μm diameter pore membrane (Costar) was coated with 200 μL Matrigel at 200 μg/mL and incubated overnight. Twenty-thousand HCCLM3 cells were seeded into the upper chamber of the Transwell and the lower chamber was filled with 0.8 mL NIH-3T3 conditioned medium or DMEM supplemented with ATX to induce chemotaxis. After 24 h of incubation at 37°C, the cells were fixed in methanol and stained with H&E, and the cells that invaded through the pores to the lower surface of the filter were counted under a microscope. Three invasion chambers were used per condition. The values obtained were calculated by averaging the total number of cells from three filters. For migration assay, wound-healing assay was done. HCCLM3 cells (1 × 10^5) were seeded on 6 cm plates coated with 10 μg/mL type I collagen. The cells were incubated in 5% CO2 at 37°C for 2 h, after a rest for 2 h by replacing medium with fresh medium, retrovirus was added again. This doubt infection resulted in a 60% to 70% of transfection efficiency.
were incubated for 24 h, monolayer was then disrupted with a cell scraper (1.2 mm width), and photographs were taken at 0 and 24 h in a phase-contrast microscope (Nikon ELWD 0.3). Experiments were carried out in triplicate, and four fields of each point were recorded.

**MTT and fluorescence-activated cell sorting analysis.** HCCLM3 cells were plated in 48-well plates at 2 × 10^4 per well. After incubation for 24 h, 20 μL MTT with 5 mg/mL concentration was added to the medium and cultured for another 4 h. Then, the medium was discarded and 150 μL DMSO was added into each well, rocking for 10 min, and the absorbencies of each well were read using a Bio-Rad model 550 microplate reader at a wavelength of 490 nm. Semilogarithmic curves for cell survival were drawn by Microsoft Excel 2000 software. For fluorescence-activated cell sorting analysis, after incubation in serum-free medium for 24 h, cells were washed with PBS at 4°C twice and were modulated concentration to 1 × 10^5/mL. Annexin V/Cy5 and propidium iodide were added for incubation for 30 min. Fluorescence-activated cell sorting analysis was done on the FACS Calibur instrument (Becton Dickinson).

**Immunohistochemistry and immunofluorescence staining.** For immunohistochemistry, briefly, tissue sections of 4 μm thick were cut and baked at 60°C overnight. After heat-induced antigen retrieval in EDTA, the sections were incubated at 37°C for 1 h with specific primary antibodies used at a 1:50 dilution followed by incubation with the second antibody at 37°C for 45 min. Then, the sections were visualized by applying 3,3'-diaminobenzidine tetrahydrochloride. Negative controls were done by omitting the primary antibody, whereas RhoC overexpression confirmed by Western blotting was used as positive controls. Diffuse, moderate to strong cytoplasmic staining characterized positive expression, whereas negative expressions were devoid of any cytoplasmic staining or contained faint, equivocal staining. For immunofluorescence, cells were grown on coverslips in DMEM containing 10% fetal bovine serum. Cells were kept on serum-free medium for 24 h before they were treated with ATX (50 ng/mL) for 60 min at 37°C. After the cells were fixed in 3.7% formaldehyde solution in PBS for 30 min and permeated in acetone at -20°C for 3 min, F-actin filaments were visualized in cells using rhodamine-conjugated phalloidin (1:40 dilution in PBS) for 20 min at room temperature. Covership was mounted on a glass slide with 20 μL SlowFade reagent (Molecular Probes).

In vivo. A metastatic human HCC cell BALB/c nude mice model was established with important modifications by including the PT67 retroviral packaging cells as a constant source of retrovirus (21, 22). Briefly, after transfecting pSUPER.retro.neo retroviral expression vector into the PT67 packaging cell line, stable virus-producing PT67 cells, named as PT67+/RhoC and PT67−/RhoCNAz, respectively, were produced and clones expressing retrovirus at 1 × 10^5 colony-forming units/0.2 mL were selected. After 1 week, when a total of 1 × 10^3 HCCLM3 cells were plated in 48-well plates at 2 × 10^5/mL. Annexin V/Cy5 and propidium iodide were added for incubation for 30 min. Fluorescence-activated cell sorting analysis was done on the FACS Calibur instrument (Becton Dickinson).

**Results.**

**Elevated expression of RhoC correlates with increased metastatic potential in HCC cells.** We first examined the expression of Rho-GTPases, including Rac1, Cdc42, RhoA, RhoB, and RhoC, in HepG2, MHCC97-L, and HCCLM3 cell lines were examined by quantitative reverse transcription-PCR. Protein expression of RhoC was determined in these cell lines using Western blot. Columns, mean of three separate experiments; bars, SD. Statistical differences between groups were compared using two-tailed ANOVA and t test. *P* < 0.05 was considered significant.

**Statistical analysis.** Statistical analysis was done using the SPSS (version 11.0). Results of quantitative data in this study were expressed as the mean ± SD. Statistical differences between groups were compared using two-tailed ANOVA and t test. *P* < 0.05 was considered significant.
were in agreement with the correlation we reported previously that RhoC expression correlates with the metastatic potential in HCC patients.

**Knockdown expression of RhoC by retrovirus-delivered siRNA in HCCLM3 cells.** To define the correlation of RhoC expression and HCC metastasis, we employed the siRNA approach to inhibit the expression of RhoC. We designed three candidate sequences and one control sequence. The forward and reverse strands of 60-bp oligonucleotides that contain the candidate siRNA-expressing sequence were annealed and cloned into the pSUPER.retro.neo retroviral expression vector (Fig. 2A). Western blot was done to assess the inhibition efficiency. As shown in Fig. 2B, the candidate sequence 2 inhibited the protein expression of RhoC by >60%, but other two candidates, sequences 1 and 3, only resulted in 30% to 40% inhibition efficiency. Together with the finding that the control sequences did not cause any detectable changes of RhoC expression, the results indicated the candidate sequence 2 as the best RhoC siRNA sequence. After 3-week selection in medium supplemented with G418, HCCLM3 cell lines, stably expressing the RhoC siRNA (sequence 2) or control siRNA (control sequence), were obtained and named as HCCLM3RhoCRNAi1 and HCCLM3RhoCRNAi2, respectively. HCCLM3 cell lines stably expressing the RhoC siRNA sequences 1 and 3 were obtained and named as HCCLM3RhoCRNAi1 and HCCLM3RhoCRNAi2, respectively. The expression of RhoC protein decreased dramatically in HCCLM3RhoCRNAi1 compared with HCCLM3RhoCRNAi2, which showed a >80% inhibitory efficiency. As expected, less effect was seen in HCCLM3RhoCRNAi3 and HCCLM3RhoCRNAi4 (Fig. 2C). To examine the specificity of RhoC siRNA, we analyzed the mRNA expression of Rac1, Cdc42, RhoA, RhoB, and RhoC in HCCLM3RhoCRNAi1 and HCCLM3RhoCRNAi2, respectively. The expression of Rac1, Cdc42, and RhoB remained unchanged (P > 0.05), which confirmed the specificity of RhoC siRNA, notwithstanding slightly up-regulated of RhoA in mRNA level was observed (P < 0.05; Fig. 2D).

**RhoC affects invasion and migration but not proliferation and apoptosis of HCCLM3 cells.** The importance of Rho proteins in the regulation of cell motility in normal cells and the association of up-regulation of RhoC with metastasis of HCC suggest that RhoC may be involved in the invasive phenotype of HCC cell through promoting cell migration. To test this possibility, we first investigated the effect of RhoC on invasion and migration of HCCLM3 cells. Wound-healing assay was employed to determine the migration of HCCLM3 cells. As shown in Fig. 3A, HCCLM3RhoCRNAi1 cells exhibited a 15% decrease in closure compared with HCCLM3RhoCRNAi2 (P < 0.05), suggesting a role for RhoC in the migration of HCCLM3 cells. To substantiate this observation, a Matrigel

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**Fig. 2.** Knockout expression of RhoC by retrovirus-delivered siRNA in HCCLM3 cells. A, schematic drawing of the construction for RNA interference retroviral expression vector. The 60-bp oligonucleotides, containing the unique 19-bp sequence in both sense and antisense orientations, separated by a 9-bp spacer sequence, were synthesized in the forward and reverse orientations, annealed, and ligated into the pSUPER.retro.neo vector excised by HindIII and BglII incision enzymes. Note the candidate sequence 2. B, expression of RhoC protein in infected HCCLM3 cells was measured to evaluate the inhibition efficiency of the three candidate sequences and the control sequence. C, after 3 wk of selection in medium supplied with G418, HCCLM3 cell lines, stably expressing siRNA-expressing sequence 2 and control sequences, were obtained and named as HCCLM3RhoCRNAi1 and HCCLM3RhoCRNAi2, respectively. HCCLM3 cell lines stably expressing the RhoC siRNA sequences 1 and 3 were also obtained and named as HCCLM3RhoCRNAi1 and HCCLM3RhoCRNAi2, respectively. Protein expression of RhoC was determined by Western blot in HCCLM3RhoCRNAi1, HCCLM3RhoCRNAi2, HCCLM3RhoCRNAi3, and HCCLM3RhoCRNAi4, respectively. D, mRNA expression of Rac1, Cdc42, RhoA, RhoB, and RhoC in HCCLM3RhoCRNAi1 and HCCLM3RhoCRNAi2 was measured by quantitative reverse transcription-PCR, respectively. Columns, mean of three separate experiments; bars, SD.
invasion assay in Transwell culture chambers was done to
determine the effect of RhoC on the in vitro invasion of
HCCLM3 cells. As shown in Fig. 3B, the number of
HCCLM3RhoC\textsuperscript{RNAi+} cells that passed through Matrigel was
only 49% compared with HCCLM3RhoC\textsuperscript{RNAi-} cells ($P < 0.05$).
More importantly, a clear correlation of different levels of
RhoC expression with the ability of HCC cells to invade was
observed among the different clones. Together, these results
support a critical role for RhoC in the invasion of HCCLM3
cells.

As cell proliferation and apoptosis could affect their ability
of migration and invasion, it is possible that the reduced ability of
wound closure and the number of cells passed through Matrigel
in HCCLM3RhoC\textsuperscript{RNAi+} cells were caused by the inhibition of
proliferation and promotion of apoptosis. Thus, we compared
HCCLM3RhoC\textsuperscript{RNAi+} and HCCLM3RhoC\textsuperscript{RNAi-} cells for cell survival
using the MTT method. As shown in Fig. 3C, a very subtle
inhibition of proliferation was observed in HCCLM3RhoC\textsuperscript{RNAi-} cells,
which counts a reduction of only 8% at day 3 and 11% at
day 5 compared with HCCLM3RhoC\textsuperscript{RNAi-} cells ($P > 0.05$).
Apoptosis in HCCLM3RhoC\textsuperscript{RNAi+} and HCCLM3RhoC\textsuperscript{RNAi-} cells
under serum starvation condition, as determined using
fluorescence-activated cell sorting analysis, showed no significant
difference ($P > 0.05$; Fig. 3D). These results suggest that RhoC

Fig. 3. RhoC affects invasion and migration but not proliferation and apoptosis of HCCLM3 cells. A, HCCLM3 cells ($1 \times 10^6$) were seeded on 6 cm plates coated with
10 \mu g/mL type I collagen. The cells were incubated for 24 h, monolayer was then disrupted with a cell scraper (1.2 mm width), and photographs were taken at 0 and 24 h in a
phase-contrast microscope. Experiments were carried out in triplicate, and four fields of each plate were recorded. B, HCCLM3 cells ($2 \times 10^5$) were seeded into the upper
chamber of the Transwell and the lower chamber was filled with 0.8 mL NIH-3T3 conditioned medium to induce chemotaxis. After 24 h of incubation at 37 °C, the cells that
invasion through the pores to the lower surface of the filter were counted under a microscope. Three invasion chambers were used per condition. The values obtained were
calculated by averaging the total number of cells from three filters. C, MTT assay was done to investigate proliferation of HCCLM3RhoC\textsuperscript{RNAi+} and HCCLM3RhoC\textsuperscript{RNAi-} cells.
Every 24 h, the absorbencies of test well were read and semilogarithmic curves were drawn. Points, mean of three separate experiments; bars, SD. D, for fluorescence-
activated cell sorting analysis, cells were incubated in serum-free medium for 24 h. Annexin V/Cy5 and propidium iodide were added for incubation for 30 min. All experiments
were repeated thrice with similar results. Columns, mean of three separate experiments; bars, SD.
contributes to metastasis without significantly effecting on proliferation and apoptosis in HCCLM3 cells.

**RhoC is essential for invasion of HCCLM3 cells induced by ATX.** Recent studies indicated that tumor cells secrete and respond to ATX in an autocrine fashion with an increased cell motility, which contributes to metastasis of tumor cells. To examine a potential role for RhoC in ATX-induced metastasis, we first assessed whether HCCLM3 cells would respond to recombinant human ATX. A Matrigel invasion assay in Transwell culture chambers was done to detect the response of HCCLM3 cells to ATX. As shown in Fig. 4A, a dose-dependent cell motility-stimulating activity of ATX was observed, with the cell motility reaching saturation in the present of ATX (50 ng/mL). To further confirm this response, GTPase pull-down assay was done to analyze the GTP-bound form of RhoC GTPases in response to ATX. The results presented in Fig. 4B indicate an activation of RhoC by ATX. Active RhoC protein level was increased by 42% in response to ATX (50 ng/mL) compared with the control group (81% versus 39%, respectively; \( P < 0.05 \)). We then examined the pattern and morphology of F-actin to determine cytoskeletal and morphologic changes induced by ATX. As shown in Fig. 4C, ATX at 50 ng/mL concentration stimulated reorganization of actin leading to the formation of stress fiber-like structures in HCCLM3RhoC\(^{RNAi+}\) cells, whereas such a process was not evident in HCCLM3RhoC\(^{RNAi-}\) cells. Furthermore, we investigated whether the migration of HCCLM3 cells induced by ATX could be affected through inhibition of RhoC. As shown in Fig. 4D, invasion and migration of HCCLM3RhoC\(^{RNAi-}\) cells were dramatically reduced when compared with HCCLM3RhoC\(^{RNAi+}\) cells in response to ATX (\( P < 0.05 \)), supporting an important role for RhoC in mediating ATX-induced invasion of HCCLM3 cells.

![Fig. 4.](image-url)
Knockdown RhoC expression via a retroviral gene delivery system inhibits the metastasis of HCCLM3 cells in vivo. To investigate whether RhoC gene could serve as a potential target to inhibit metastasis of HCC, we established an anti-RhoC retroviral gene delivery BALB/c nude mice model as described previously (24, 25). After 1 week, when HCCLM3 cells were s.c. transplanted to generate primary HCC tumor, PT67 cells (PT67RhoCRNAi+ or PT67RhoCRNAi−), with high titer about 10^5, were inoculated s.c. as a constant source of retrovirus, these nude mice models were named as MiceRhoCRNAi+ and MiceRhoCRNAi−, respectively. Primary s.c. tumor of HCCLM3 was measured every 5 days and mice were sacrificed 45 days after implantation to examine lung metastasis. Infection of HCCLM3 cells was confirmed first through histologic examination of green fluorescent protein expression. HCCLM3 tumor showed strong expression of green fluorescent protein, indicating a successful retroviral gene delivery. At the same time, the expression of RhoC in HCCLM3 tumor was also determined to assess the effect of siRNA against RhoC in vivo. As expected, expression of RhoC in primary HCCLM3 tumor was negative in MiceRhoCRNAi+ but positive in MiceRhoCRNAi−, which indicated a successful knockdown the expression of RhoC in HCCLM3 cells in vivo (Fig. 5A). Next, the primary tumor size and the number of lung metastasis were assessed. Significantly, the numbers of lung metastasis, identified and counted by pathologist under microscope, were significantly fewer in MiceRhoCRNAi+ than in MiceRhoCRNAi− (P < 0.01; Fig. 5B). Meanwhile, a difference in the primary tumor size was detected. The primary tumor was smaller in MiceRhoCRNAi+ than in MiceRhoCRNAi− (P < 0.05), which suggested that knockdown of RhoC in vivo could inhibit growth of HCC (Fig. 5C). Finally, MVD in primary tumor was investigated. Primary tumor in MiceRhoCRNAi+ showed significantly less MVD than MiceRhoCRNAi− (P < 0.05), suggesting that knockdown of RhoC in vivo may inhibit growth of HCC through inhibition of angiogenesis.

Discussion

Given the role of Rho-GTPases in the regulation of cell motility in normal cells and their aberrant regulation in tumor cells, it is thought that they may be involved in the invasive phenotype of tumor cells (6, 13). Recent studies have indeed indicated the essential role of RhoC in metastasis of inflammatory breast cancer and melanoma. In our previous study, we reported a correlation between overexpression of RhoC and metastasis of HCC in clinical specimens (14–16). In this study, we first confirmed this correlation in three HCC cell lines with differently spontaneous metastatic potential, HepG2, MHCC97-L, and HCCLM3. HepG2 cells exhibited a moderate metastatic potential, whereas HCCLM3 cells were highly invasive as shown by extensive metastases via both s.c. and orthotopic inoculation (26). In agreement with the difference in metastasis potentials, expression of RhoC, in both mRNA and protein levels, was significantly higher in HCCLM3 cell line when compared with HepG2 cell line and MHCC97-L. As previous study has shown that this cell model system can serve as a useful platform for the study of HCC metastasis (24, 25). Our results obtained from this system suggested an association of up-regulation of RhoC with the metastasis potential of HCC. To further examine the importance of RhoC in HCC metastasis, we employed siRNA to knockdown specifically the expression of RhoC in HCC cells. Other Rho-GTPases, including Rac1, Cdc42, RhoA, and RhoB, were not significantly affected, except only a slightly up-regulated of RhoA supporting the specificity of this method. A similar result was reported in breast carcinoma SUM-159 cell line, which revealed a compensatory relationship between RhoA and RhoC (27). As two Rho isoforms, RhoA and RhoC, share high sequence similarity, it is possible that they share similar feedback regulation or signaling pathway, which resulted in this compensation (28).

Here, we showed that knockdown of RhoC is associated with the inhibition of invasion and migration but not proliferation and apoptosis of HCCLM3 cells, consistent with the critical role of RhoC in control of cellular motility (6). To further substantiate the importance of RhoC in HCC metastasis, we assessed its role in ATX-induced migration in HCCLM3 cells. ATX, an exonucleotide pyrophosphatase and phosphodiesterase, was originally isolated as a potent stimulator of tumor cell motility (29). ATX appears to augment cellular characteristics necessary for tumor aggressiveness. Previous studies have reported the overexpression of ATX in HCC. Increasing evidence indicated ATX as a tumor-secreted autocrine motility-stimulating factor that has been associated with tumor invasion and metastatic potential (30–32). In the present study, ATX was added in medium as a chemotaxin and dramatically increased the invasion and migration of HCCLM3RhoCRNAi− cells. Such an effect of ATX was significantly diminished in HCCLM3RhoCRNAi+ cells, implicating a critical role of RhoC in ATX signal pathway and HCC metastasis.

To examine the role of RhoC in metastasis for its in vivo relevance, we tested whether RhoC could be a potential target to inhibit metastasis of HCC. To accomplish this, we adopted the metastatic human HCC cell nude mice model (23, 25, 33), with important modifications as described by Hoang et al. (22). In this model, the PT67 cell line, with high titer about 10^5, was inoculated s.c. as a constant source of retrovirus. Although retroviral delivery of siRNA in tumors may have potential risk for its clinical application (34), there are two major advantages for this retroviral gene delivery system compared with other systems, especially in our present study. First, retroviral gene transfer can lead to stable integration of gene of interest into the target cell genome, thus providing the long-term gene silence. Second, retroviral-mediated gene transfer is limited to the transduction of dividing cell; the obvious advantage is that most of normal cells, such as muscle cells, liver cells, and fibroblasts, are not actively dividing, whereas tumor cells inoculated s.c. were preferentially infected because of high proliferation rate. In the present study, infection of tumor cells was confirmed through histologic examination of green fluorescent protein expression and the expression of RhoC in HCCLM3 tumors. Our results indicated a successful gene delivery in mice. Most importantly, our results proved that knockdown RhoC expression could inhibit metastasis of HCC in vivo for the first time. Especially, the inhibition can be achieved after inoculated tumor has been developed. Interestingly, the in vivo results showed that knockdown the expression of RhoC inhibited proliferation of primary tumor of HCC, which differs from the results in vitro. We hypothesized that it is the
microenvironment, for instance, angiogenesis, which may be responsible for this difference. Therefore, MVD in primary tumor of HCC were investigated. Indeed, our result showed a less MVD in RhoC deficiency HCC tumor, suggesting that knockdown of RhoC in vivo can inhibit growth of HCC through inhibition of angiogenesis. In fact, it has been reported that overexpression of RhoC GTPase is specifically and directly implicated in the control of production of angiogenic factors by inflammatory breast cancer cells (35, 36). Recently, evidence also indicated that RhoC-induced cytoskeletal changes and the release of vascular permeability factors work cooperatively to mediate cell intravasation during

**Fig. 5.** Knockout RhoC expression via a retroviral gene delivery system inhibits the metastasis of HCCLM3 cells in vivo. A, preparation of anti-RhoC retroviral gene delivery BALB/c nude mice model. After 1 wk, when HCCLM3 cells were s.c. transplanted in right flank (black arrows), PT67RhoCRNAi- or PT67RhoCRNAi+ cells were inoculated s.c. in left flank (white arrow) as a constant source of retrovirus (a and b). Immunohistochemistry examination of green fluorescent protein and RhoC expression in HCCLM3 tumor was done in MiceRhoCRNAi- (c and e) and MiceRhoCRNAi+ (d and f). B, metastasis in H&E-stained lung sections from MiceRhoCRNAi- and MiceRhoCRNAi+. Arrows, lung metastasis, ×40 (a and c) and ×400 (b and d). Columns, mean number of metastasis per lung (n = 10); bars, SD (e). C, primary tumor size was investigated in MiceRhoCRNAi- and MiceRhoCRNAi+ every 5 d after cell implantation. The tumors shown are from the final time point. Points, mean (n = 10); bars, SD. D, MVD (arrows) was assessed by CD31 immunolabeling on paraffin-embedded HCCLM3 tumor sections. Representative examples of results obtain in MiceRhoCRNAi- (a) and MiceRhoCRNAi+ (b). Columns, mean (n = 10); bars, SD (c).
the early stages of cancer cell metastasis (37). Further investigation of the role of RhoC in angiogenesis of HCC is needed.

In summary, the present study provides evidence indicating a critical and specific role of RhoC in metastasis of HCC. Furthermore, data from our present study implicate a new cancer therapy that blocks HCC metastasis by specifically inhibiting RhoC.

Disclosure of Potential Conflicts of Interest

No potential conflicts of interest were disclosed.

Acknowledgments

We thank Dr. Cao Yu's laboratory (Cancer Research Institute, Xiangya Medicine School, Central South University) for technical assistance.

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Inhibition of Invasion and Metastasis of Hepatocellular Carcinoma Cells via Targeting RhoC  *In vitro* and *In vivo*

Wei Wang, Fan Wu, Feng Fang, et al.

*Clin Cancer Res* 2008;14:6804-6812.

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