RhoC is essential for angiogenesis induced by hepatocellular carcinoma cells via regulation of endothelial cell organization

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The angiogenesis induced by tumor cells is essential for metastasis of hepatocellular carcinoma. Available information suggests that RhoC participates in angiogenesis through regulation of vascular endothelial growth factor expression in tumor cells. For its broad functions in cell migration and cytoskeletal organization, we hypothesized that RhoC regulating angiogenesis does not exclusively depend on regulation of vascular endothelial growth factor expression. To address this question, in the present study, we used a retroviral small interfering RNA approach to selectively knockdown the expression of RhoC in a neovascularization model *in vivo* and *in vitro*. Our present results indicate that RhoC is the downstream regulator of vascular endothelial growth factor in endothelial cells and is essential for angiogenesis induced by vascular endothelial growth factor, notwithstanding that RhoC regulates the expression of vascular endothelial growth factor in tumor cells. Furthermore, we show that knockdown of RhoC is associated with the inhibition of invasion and migration but not apoptosis of endothelial cells. Knockdown of RhoC results in inhibition of endothelial cell organization through restraining the reorganization of F-actin filaments, which represses endothelial cell network and sprout formation. In conclusion, our results demonstrate that knockdown of RhoC inhibits angiogenesis induced by tumor cells not only through effecting the release of vascular endothelial growth factor, but also through inhibiting endothelial cell migration and organization, which implies that it blocks tumor metastasis by specifically inhibiting RhoC in endothelial cells. (*Cancer Sci* 2008; 99: 2012–2018)

Metastasis is the major cause of death in hepatocellular carcinoma (HCC) patients, and there are currently no therapeutic agents available to prevent this disease. The metastasis of HCC is a multistep process that often involves many complex biological and pathological events.[1] Available information suggests that the metastatic process is to a large extent attributable to angiogenesis induced by tumor cells. However, this complex process remains incompletely understood.[2] Thus, there is a critical need to understand the invasive mechanisms and angiogenic programs that facilitate metastasis so that therapeutic strategies can be developed to prevent HCC progression.

Rho-GTPases, which are members of the Ras superfamily of small GTPases, shuttle between an inactive GDP-bound and an active GTP-bound form and exhibit intrinsic GTPase activities. Activation of Rho protein leads to assembly of actin–myosin contractile filaments into focal adhesion complexes that lead to cell polarity and facilitate motility.[3] More recently, RhoC has attracted interest with its increased expression being linked to increased invasion and metastasis in breast cancer, melanoma, pancreatic cancer, colon cancer, bladder cancer, HCC, non-small cell lung carcinoma, and primary gastric tumors and cell lines.[4–10] Our previous study provides evidence indicating a critical and specific role for RhoC in the metastasis of HCC through regulating the invasion and migration of HCC cells. Recent evidence also indicates that RhoGTPases are part of the signaling pathways triggered by angiogenic inducers and regulate the shape of endothelial cells. Overexpression of RhoC GTPase is specifically and directly implicated in the control of the production of angiogenic factors by inflammatory breast cancer cells.[11,12] RhoC-induced cytoskeletal changes and the release of vascular permeability factors work cooperatively to mediate cell intravasation during the early stages of cancer cell metastasis.[13] These studies suggest that release of vascular endothelial growth factor (VEGF) by invasive tumor cells disrupts endothelial cell–cell connections, which creates vascular disruptions that serve as portholes for cell intravasation. However, the detailed mechanism of how RhoC regulates angiogenesis remains incompletely understood and further investigation is anticipated.

Morphogenesis, the essential step for angiogenesis, is characterized mainly by cell motility and shape rearrangement, which imply dramatic changes in endothelial cell cytoskeletal dynamics. The endothelial cells migrate into the perivascular space toward chemotactic angiogenic stimuli and form a migration column; these are the critical processes during angiogenesis.[14,15] As the RhoGTPases regulate a wide array of cellular processes, including cell migration and cytoskeletal organization,[16] we hypothesized that RhoC regulates angiogenesis not only through effecting release of VEGF, but also through regulating the migration of endothelial cells. To address these questions in the present study, we used a retroviral small interfering RNA (siRNA) approach to selectively knockdown the expression of RhoC *in vivo* and *in vitro*, which allowed us to investigate the mechanism of RhoC regulation of angiogenesis.

Materials and Methods

Antibodies and reagents. Purified recombinant human $VEGF_{165}$ was purchased from Chemicon (Temecula, CA, USA). Goat anti-RhoC antibody and goat anti-VEGF antibody were purchased from Santa Cruz Biotechnology (Santa Cruz, CA, USA). Biotin antimouse CD31 antibody was purchased from Biolegend (San Diego, CA, USA). Rhodamine-conjugated phalloidin was purchased from Molecular Probes (Eugene, OR, USA). pSUPER.retro.neo retroviral expression vector was purchased from Oligoengine (Seattle, WA, USA). Matrigel was purchased from Becton-Dickinson (Franklin Lakes, NJ, USA). Cytodex-3 microcarriers were purchased from Amersham Pharmacia Biotech (Piscataway, NJ, USA).

Cell culture and transfection. HMEC-1, a human dermal microvascular endothelial cell line, was purchased from the American Type Culture Collection (Rockville, MD, USA). HCCLM3, a HCC cell line with high metastatic potential, was obtained from

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the Liver Cancer Institute and Zhong Shan Hospital of Fudan University, Shanghai, China. The PT67 retroviral packaging cell line was purchased from BD Biosciences Clontech (Palo Alto, CA, USA). The HCCLM3 and PT67 cell lines were cultured in high-glucose Dulbecco's modified eagle medium (DMEM) supplemented with 10% fetal bovine serum (FBS) and incubated in 5% CO_2 at 37°C, whereas the HMEC-1 cell line was cultured in low-glucose DMEM supplemented with 10% FBS and incubated under the same conditions. For transfection of packaging cells, the PT67 retroviral packaging cells, which express the 10A1 viral envelope for production of amphotropic virus, were transfected with the relevant reconstructed pSUPER.retro.neo retroviral expression plasmid at 70% confluence with the use of Lipofectamine 2000 (Invitrogen, Carlsbad, CA, USA) according to the manufacturer's instructions. Clones expressing retrovirus at 1×10^5 colony-forming units (cfu)/mL were selected for subsequent experiments. Multiple rounds of infection, which can increase the number of infected cells as well as increase the number of copies per cell, were carried out during the transfection of HMEC-1 cells. Briefly, media containing retrovirus was added to the cells supplied with Polybrene (Sigma-Aldrich, St Louis, MO, USA) (8 μg/mL). After 24 h, the medium was replaced with fresh medium and the retrovirus was added again. Infecting the cells three times resulted in an 80–90% transfection efficiency.

Generation of siRNA constructs. Reconstructed pSUPER.retro.neo retroviral expression plasmid, containing siRNA directed against human RhoC plasmid, was prepared previously in our laboratory. Briefly, the 60-bp oligonucleotides, containing the unique 19-bp RhoC siRNA sequence in both sense and antisense orientations separated by a 9-bp spacer sequence, were synthesized in the forward and reverse orientation, annealed, and ligated into the pSUPER.Retro.neo vector excised by the *Hand*III and *Bgl*II incision enzymes. The presence of the insert was determined by sequencing. The effective sequence and control sequence were as follows: effective sequence sense, 5′-GATCCCCCATGTGCTTCTCCATCGACTTCAAGAGAGTCGATGGAGAAGCACATGTTTTTA-3′; effective sequence antisense, 5′-AGCTTAAAAACATGTGCTTCTCCATCGACTCTCTTGAAGTCGATGGAGAAGCACATGGGG-3′; control sequence sense, 5′-GATCCCCCAGTCAGGAGGATCCAAAGTTCAAGAGACTTTGGATCCTCCTGACTGTTTTTA-3′; and control sequence antisense, 5′-AGCTTAAAAACAGTCAGGAGGATCCAAAGTCTCTTGAACTTTGGATCCTCCTGACTGGGG-3′.

Western blotting. Cells were lysed in a lysis buffer (Pierce, Rockford, IL, USA). 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 antibody, diluted at 1:500; goat anti-VEGF antibody, diluted at 1:2000), followed by incubation with a 1:1000 dilution of horseradish peroxidase-linked rabbit antigoat antibody (Santa Cruz Biotechnology). The membrane was then washed and treated with western blotting luminal reagent (Pierce) to visualize the bands.

Invasion and migration assays. For the invasion assay, a transwell with an 8-μm diameter pore membrane (Costar, Cambridge, MA, USA) was coated with 200 μL matrigel at 200 μg/mL (Becton-Dickinson) and incubated overnight. Twenty thousand HMEC-1 cells were seeded into the upper chamber of the transwell and the lower chamber was filled with 0.8 mL low-glucose DMEM supplemented with VEGF (10 ng/mL). After 24 h of incubation at 37°C, the cells were fixed in methanol and stained with hematoxylin–eosin 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 the migration assay, a wound-healing assay was carried out. HMEC-1 cells (1×10^6) were seeded on 6-cm plates coated with 10 μg/mL type I collagen. The cells were incubated for 24 h before the monolayer was disrupted with a cell scraper (1.2-mm width). Photographs were taken at 0 and 24 h using a phase-contrast microscope (ELWD 0.3; Nikon, Tokyo, Japan). Experiments were carried out in triplicate, and four fields were recorded for each point.

FACS analysis. For fluorescence-activated cell sorter (FACS) analysis, after incubation in serum-free media for 24 h, cells were washed twice with phosphate-buffered saline at 4°C, and the concentration was adjusted to 1×10^6/mL. Annexin V/Cy5 and propidium iodide (PI) were added and incubated for 30 min. FACS analysis was carried out using a FACS caliber instrument (Becton Dickinson). Experiments were repeated three times with similar results.

Immunohistochemistry and immunofluorescence staining. For immunohistochemistry, briefly, tissue sections of 4 μm in thickness were cut and baked at 60°C overnight. After heat-induced antigen retrieval in ethylenediaminetetraacetic acid buffer, 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 secondary antibody at 37°C for 45 min. The sections were then visualized by applying 3,3-diaminobenzidine tetrahydrochloride. A negative control omitted the primary antibody, whereas overexpression of the target protein confirmed by western blotting was used as a positive control. Diffuse, moderate to strong cytoplasmic staining characterized positive expression, whereas negative expression was devoid of any cytoplasmic staining or contained faint, equivocal staining. For immunofluorescence, cells were grown on matrigel in DMEM containing 10% FBS for 18 h, then F-actin filaments were visualized in cells using rhodamine-conjugated phalloidin (1:40 dilution in phosphate-buffered saline) for 20 min at room temperature.

In vivo. A RhoC-RNAi (RNA interference) retroviral gene-delivery BALB/c nude mouse model was established with important modifications by including the PT67 retroviral packaging cells as a constant source of retrovirus.[17,18] Briefly, after transfecting the reconstructed pSUPER.retro.neo retroviral expression plasmid into the PT67 packaging cell line, stable virus-producing PT67 cells, named $PT67^{RhoCRNAi+}$ and $PT67^{RhoCRNAi-}$ (control group), respectively, were produced and clones expressing retrovirus at 1×10^5 cfu/mL were selected. After 1 week, a total of 1×10^7 HCCLM3 cells in 0.2 mL DMEM were transplanted subcutaneously into the right flank of BALB/c nude mice, and 5×10^6 retroviral packaging cells ($PT67^{RhoCRNAi+}$ or $PT67^{RhoCRNAi-}$), with a high titer of approximately 10^5, were inoculated subcutaneously into the left flank as a constant source of retrovirus. Mice were killed 45 days after implantation and the tumors were removed and fixed in 10% formalin, embedded in paraffin and cut into 4 μm-thick slices for standard histopathological study. Paraffin sections embedded with the subcutaneous tumor were stained with hematoxylin–eosin for histological examination and stained with anti-green fluorescent protein (GFP) antibody or anti-RhoC antibody for evaluating infection efficiency or suppression efficiency *in vivo*. For evaluating microvessel density (MVD), paraffin sections were stained with antimouse CD31 antibody, then three areas with the most vessels within each section were selected for quantitation of angiogenesis, and vessels labeled with the antimouse CD31 antibody were counted under a light microscope at ×200 magnification. The histopathological examination was carried out by a pathologist who was masked from the experimental group designation. For the angiogenesis assay *in vivo*, based on the RhoC-RNAi retroviral gene-delivery BALB/c nude mice model, we developed a neovascularization model *in vivo*, in which PT67 packing cells were inoculated subcutaneously as a constant source of retrovirus, and matrigel mixed with VEGF was inoculated subcutaneously to drive angiogenesis. One week after implantation, matrigel (including skin) was dissected and photographed under a visible-light microscope (×20). New blood vessels on the

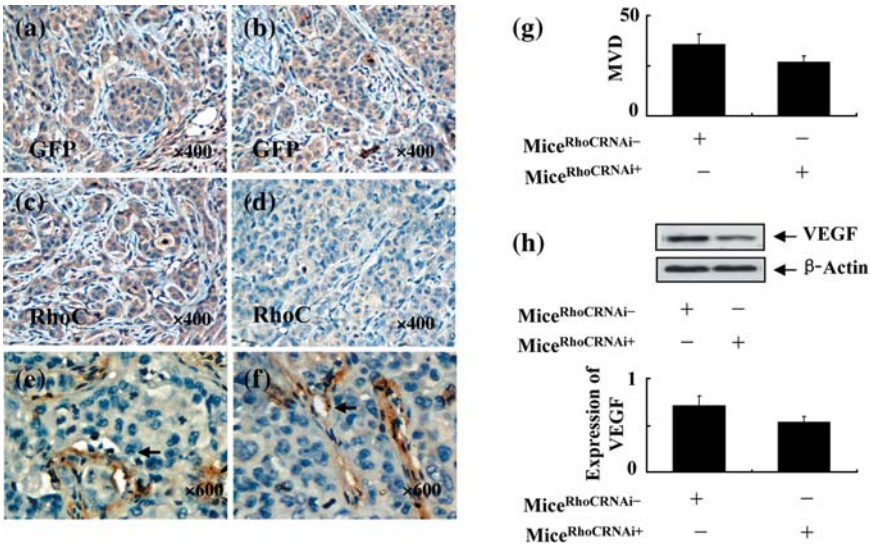

Fig. 1. RhoC is essential for angiogenesis induced by hepatocellular carcinoma cells. A RhoC-RNAi (RNA interference) retroviral gene-delivery BALB/c nude mouse model was established as described previously. Immunohistochemical examination of green fluorescent protein (GFP) and RhoC expression in HCCLM3 tumor cells was carried out in (a,c) mice$^{RhoCRNAi–}$ and (b,d) mice$^{RhoCRNAi+}$. Meanwhile, microvessel density (MVD) (arrows) was assessed by CD31 immunolabeling on paraffin-embedded HCCLM3 tumor sections. Photographs are representative examples of results obtain in (e) mice$^{RhoCRNAi–}$ and (f) mice$^{RhoCRNAi+}$. (g) Results are shown as the mean ± SD. Columns, mean (n = 10); bar, SD. (h) The expression of vascular endothelial growth factor protein in HCCLM3 tumor cells was examined by western blotting and the results are shown as the mean ± SD. Columns, mean (n = 10); bar, SD.

matrigel were counted and their lengths were determined.[19] Sterile small-animal surgical techniques were followed during the whole procedure. All experimental protocols were approved by the Institutional Animal Care and Use Committee, Xiangya Hospital, Central South University.

***In vitro* angiogenesis: endothelial cell network formation assays.** To analyze *in vitro* angiogenesis, a 24-well tissue culture plate was coated with 0.2 mL matrigel (8.8 mg/mL). HMEC-1 cells (1×10^5 cells/mL) were seeded on matrigel and incubated at 37°C in a 5% CO_2 humidified atmosphere for 18 h. The network formation was observed under an inverted phase-contrast microscope (Nikon), and the images were captured with a video graphic system (Retiga 1300 Digital Output Camera; QIMAGING, Surrey, BC, Canada). The number of network projections in 10 × 40 fields was counted for three independent experiments in each group, and the data were obtained using the following formula:

$$\text{angiogenesis} = a \times b,$$

where a = the number of branch points per ×40 field and b = the total number of branches per point.[19]

***In vitro* angiogenesis: endothelial cell sprout formation assays.** HMEC-1 cells were mixed with Cytodex-3 microcarriers (Amersham Pharmacia Biotech) at a concentration of 300 HMEC-1 cells per bead in 1 mL DMEM medium. Beads with cells were shaken gently every 20 min for 4 h at 37°C and 5% CO_2. After incubation, beads with cells were transferred to a six-well tissue culture plate (BD Biosciences, Bedford, MA, USA) and left for 12–16 h in 1.5 mL DMEM at 37°C and 5% CO_2. The following day, beads with cells were washed three times and resuspended at a concentration of 200 cell-coated beads/mL in 4 mg/mL matrigel. Next, 0.8 mL matrigel mixed with cell-coated beads was add to one well of a 24-well tissue culture plate and culture for 1 week. The sprouting of HMEC-1 cell was count as described previously.[20] Briefly, high-resolution images of beads were captured on an IX70 microscope (Olympus, Tokyo, Japan) with a ×4 objective. Capturing images at low magnification has the advantage of a high depth of field, which enables inclusion of all sprouts in focus. Images were then magnified in Adobe Photoshop (Adobe Systems, San Jose, CA, USA) and analyzed using ImageJ (National Institutes of Health, Bethesda, MD, USA). The number of sprouts per bead was determined, where a sprout was defined as a vessel of length equal to the diameter of a bead. For statistical analysis, the number of sprouts/10 beans was counted and 100 beads were assessed for each condition.

Statistical analysis. Statistical analysis was carried out using SPSS software (version 11.0; SPSS, Chicago, IL, USA). The results of the quantitative data in the present study are expressed as mean ± SD. Statistical differences between groups were compared using two-tailed ANOVA and t-test. A P-value of less than 0.05 was considered significant.

Results

RhoC is essential for angiogenesis induced by HCC cells. To investigate whether RhoC is essential for angiogenesis induced by HCC cells, we established a RhoC-RNAi retroviral gene-delivery BALB/c nude mouse model as described previously.[17,18] As our reconstructed pSUPER.retro.neo retroviral expression plasmid contains GFP-expressing framework, the infection and the validity of small interference were confirmed first through histological examination of GFP and RhoC expression. The HCCLM3 tumor showed strong expression of GFP, indicative of successful retroviral gene delivery. At the same time, expression of RhoC in the HCCLM3 tumor was negative in mice$^{RhoCRNAi+}$ but positive in mice$^{RhoCRNAi–}$, which indicated the successful knockout expression of RhoC *in vivo* (Fig. 1a–d). Based on this successful nude mouse model, the MVD in the HCC tumor was investigated to assess the angiogenesis induced by HCC cells. Tumor in mice$^{RhoCRNAi+}$ showed significantly less MVD than in mice$^{RhoCRNAi–}$; the MVD was 27 ± 3 per field in mice$^{RhoCRNAi+}$ but 36 ± 5 per field in mice$^{RhoCRNAi–}$ ($P < 0.05$) (Fig. 1e,f), suggesting that knockdown of RhoC *in vivo* may inhibit angiogenesis induced by HCC cells. Meanwhile, the VEGF expression of the HCC tumor was examined; the VEGF expression of the HCC tumor was 0.47 ± 0.11 in mice$^{RhoCRNAi+}$ and 0.63 ± 0.09 in mice$^{RhoCRNAi–}$ ($P < 0.05$) (Fig. 1g), which suggests that knockdown of RhoC could inhibit VEGF expression in HCC tumors.

RhoC is essential for angiogenesis induced by VEGF. Expression of VEGF in the HCC tumor was downregulated in mice$^{RhoCRNAi+}$, indicating that RhoC regulates angiogenesis probably through downregulation of VEGF expression in HCC tumors. However, it is not known whether there is another pathway through which RhoC can regulate angiogenesis. Therefore, based on the RhoC-RNAi retroviral gene-delivery BALB/c nude mouse model, we developed a neovascularization model *in vivo*, in which PT67 packing cells were inoculated subcutaneously as a constant source of retrovirus and matrigel mixed with VEGF was inoculated subcutaneously to drive angiogenesis (Fig. 2a,b). As anticipated,

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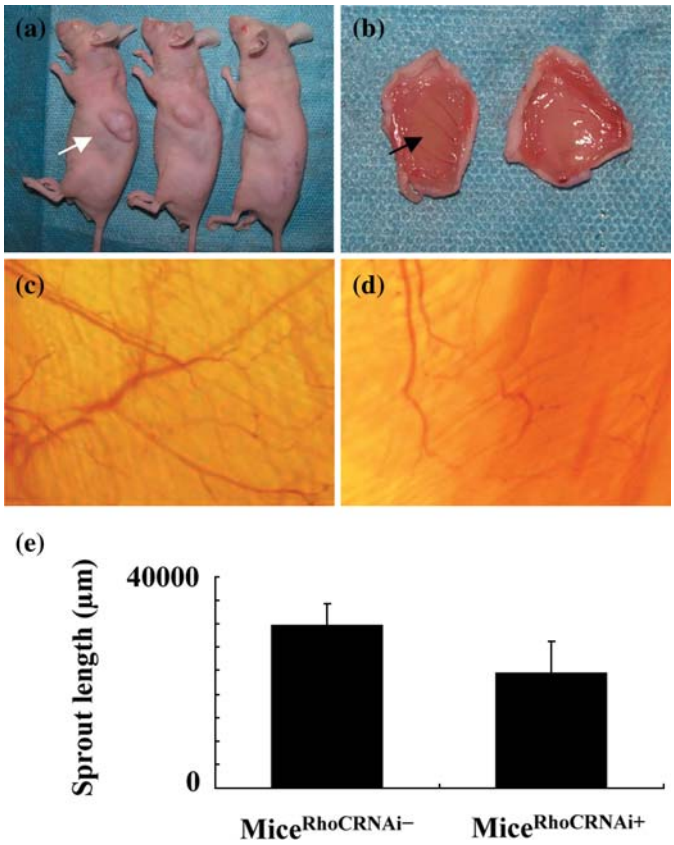

Fig. 2. RhoC is essential for angiogenesis induced by vascular endothelial growth factor (VEGF). (a) Based on the RhoC-RNAi (RNA interference) retroviral gene-delivery BALB/c nude mouse model, we developed a neovascularization model *in vivo*, in which PT67 packing cells were inoculated subcutaneously as a constant source of retrovirus (white arrow) and matrigel mixed with VEGF was inoculated subcutaneously to drive angiogenesis. (b) One week after implantation, matrigel including skin was dissected (black arrow) and photographed under a visible-light microscope (×20). (c) Mice$^{RhoCRNAi–}$; (d) mice$^{RhoCRNAi+}$. New blood vessels on the matrigel were counted and their lengths were determined. (e) Results are shown as the mean ± SD. Columns, mean (n = 10); bar, SD.

neovascularization was markedly reduced in RhoC-deficient mice. The total length of angiogenesis in matrigel was 24532 ± 6783 μm and 34869 ± 4396 μm in mice$^{RhoCRNAi+}$ and mice$^{RhoCRNAi–}$, respectively ($P < 0.05$) (Fig. 2c–e), indicating that RhoC critically regulates angiogenesis driven by VEGF *in vivo*. Meanwhile, this result also indicates that RhoC regulation of angiogenesis induced by HCC cells did not exclusively depend on downregulation of VEGF expression.

RhoC participates in invasion and migration of HMEC-1 cells. We hypothesized that RhoC plays a critical role in the migration of endothelial cells, which may be responsible for regulation of angiogenesis. We therefore investigated the effect of RhoC on the invasion and migration of HMEC-1 cells. First, the siRNA approach combining multiple rounds of infection was used to inhibit RhoC expression in HMEC-1 cells, which resulted in ~80–90% transfection efficiency. The expression of RhoC protein in HMEC-1$^{RhoCRNAi+}$ and HMEC-1$^{RhoCRNAi–}$ was 0.33 ± 0.05 and 0.84 ± 0.07, respectively ($P < 0.05$) (Fig. 3a), suggesting successful inhibition of RhoC in HMEC-1 cells. Next, a wound-healing assay was used to determine the migration of HMEC-1 cells. HMEC-1$^{RhoCRNAi+}$ decreased wound closure by 23% compared with HMEC-1$^{RhoCRNAi–}$, suggesting a role for RhoC in the migration of HMEC-1 cells ($P < 0.05$) (Fig. 3b). Meanwhile, a matrigel invasion assay in transwell culture chambers was carried out to determine the effect of RhoC on the *in vitro* invasion of HMEC-1 cells. The number of HMEC-1$^{RhoCRNAi+}$ cells that passed through the matrigel was 49 ± 6 compared with 70 ± 3 HMEC-1$^{RhoCRNAi–}$ cells ($P < 0.05$) (Fig. 3c). At the same time, apoptosis in HMEC-1$^{RhoCRNAi+}$ and HMEC-1$^{RhoCRNAi–}$ cells under serum-starvation conditions, determined using FACS, showed no significant difference ($P > 0.05$) (Fig. 3d). Together, these results support a critical role for RhoC in the invasion and migration but not apoptosis of HMEC-1 cells.

Knockdown of RhoC inhibits angiogenesis *in vitro*. As a critical role for RhoC in the migration of HMEC-1 cells has been confirmed, we hypothesized that knockdown of RhoC may inhibit angiogenesis through inhibition of HMEC-1 organization. Therefore, the effect of RhoC on HMEC-1 cell organizational behavior in matrigel was investigated. As shown in Figure 4a, inhibition of RhoC in HMEC-1 cells resulted in inhibition of capillary network formation of HMEC-1 cells in matrigel, with the index of capillary network formation being 489 ± 215 and 2397 ± 726 in HMEC-1$^{RhoCRNAi+}$ and HMEC-1$^{RhoCRNAi–}$, respectively ($P < 0.05$). Meanwhile, with the help of rhodamine-conjugated phalloidin, F-actin filaments of HMEC-1 cells in matrigel were visualized, as shown in Figure 4b. Reorganized HMEC-1 cells had more F-actin filaments than HMEC-1 cells without reorganization, indicating that RhoC regulates endothelial cell organization through reorganization of the F-actin filaments. To further confirm the critical role of RhoC in angiogenesis, HMEC-1 cells were coated on Cytodex-3 microcarriers and cultured in a three-dimensional angiogenesis model. As expected, knockdown of RhoC inhibited tube formation of HMEC-1 in three-dimensional matrigel, with the number of sprouts per 10 beads being 9 ± 7 and 45 ± 9 in HMEC-1$^{RhoCRNAi+}$ and HMEC-1$^{RhoCRNAi–}$, respectively ($P < 0.05$) (Fig. 4c). Together, these results demonstrate that knockdown of RhoC inhibits angiogenesis *in vitro*.

Discussion

We investigated the MVD in HCC tumors to assess the effect of RhoC on angiogenesis induced by HCC cells. To accomplish this, based on the established HCC cell nude mouse model,[21–23] we developed this model with important modifications as described by Hoang *et al.*[18] In this model, the PT67 cell line, with a high titer of approximately 10^5, was inoculated subcutaneously as a constant source of retrovirus. There are two major advantages for this retroviral gene-delivery system compared with other gene-delivery systems. First, retroviral gene transfer can lead to stable integration of the gene of interest into the target-cell genome, thus providing long-term gene silencing. Second, retrovirus-mediated gene transfer is limited to the transduction of dividing cells, the obvious advantage being that most normal cells, such as muscle cells, liver cells, and fibroblasts, are not usually dividing, whereas tumor cells inoculated subcutaneously during proliferation and endothelial cells during neovascularization were preferentially infected. Our present study suggests that knockdown of RhoC *in vivo* inhibits angiogenesis induced by HCC. Meanwhile, our study also indicates that knockdown of RhoC inhibits VEGF expression in HCC tumors. In fact, it has been reported that overexpression of RhoC GTPase is specifically and directly implicated in the control of the production of angiogenic factors by inflammatory breast cancer cells.[11,12] Our results confirm that RhoC regulates the expression of VEGF in tumor cells.

Angiogenesis, the essential and critical process in metastasis, begins with the migration of precursor angioblast cells to sites where they differentiate into endothelial cells. These then proliferate and migrate to form a solid cord of cells leading away larger vessels.[14] Cell migration plays a critical role during this step-wise process. Other studies have shown that the RhoGTPases serve important functions in regulating the angiogenic processors

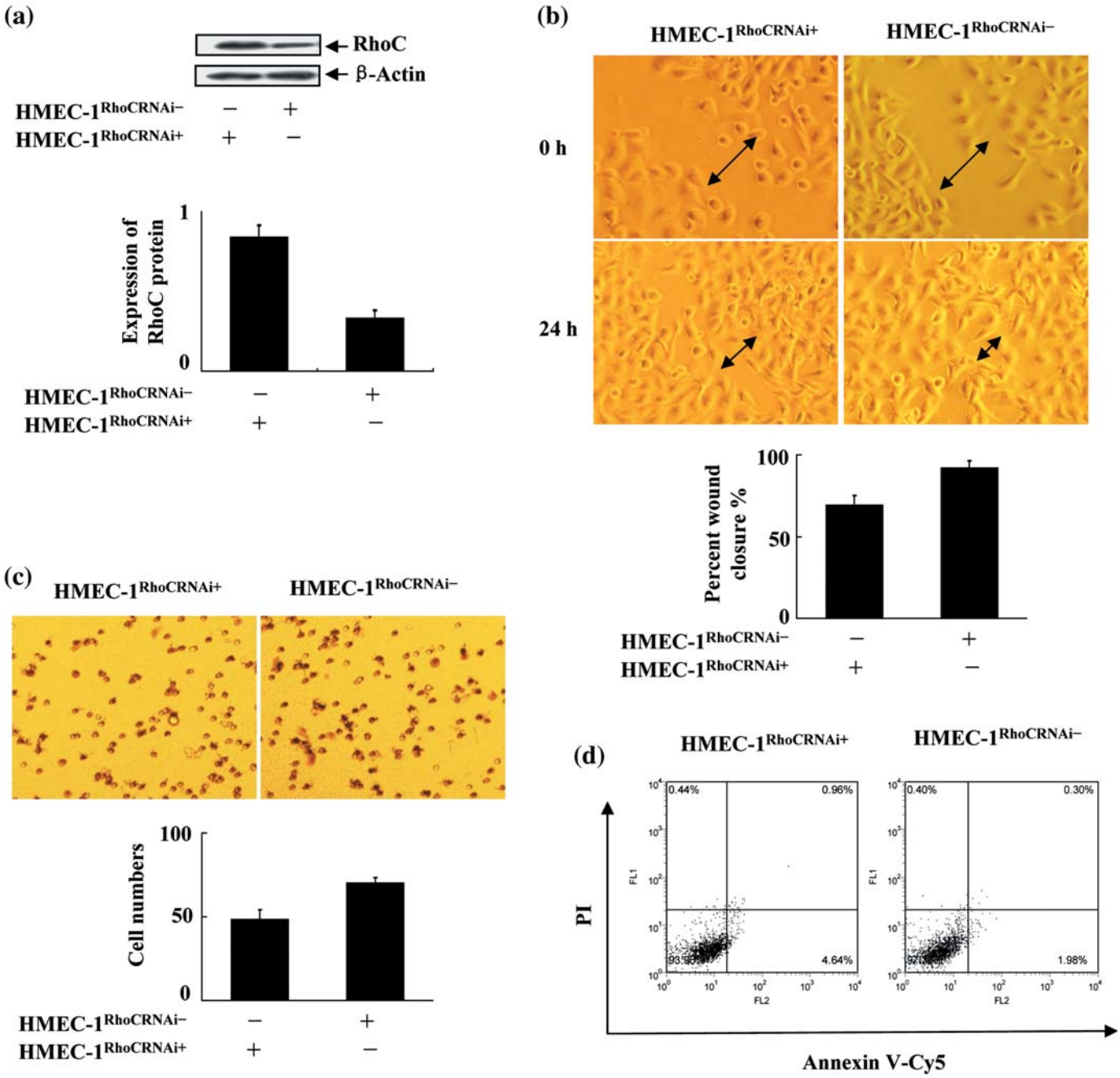

Fig. 3. RhoC participates in the invasion and migration of HMEC-1 cells. (a) The small interfering RNA approach combining multiple rounds of infection was used to inhibit the expression of RhoC in HMEC-1 cells. Expression of RhoC protein was examined to assess the inhibition efficiency. Results are shown as the mean ± SD. Columns, mean (n = 3); bar, SD. (b) HMEC-1 cells (1×10^6) were seeded on 6-cm plates coated with 10 μg/mL type I collagen. The cells were incubated for 24 h, the monolayer was then disrupted with a cell scraper (1.2-mm width), and photographs were taken at 0 and 24 h under a phase-contrast microscope. Experiments were carried out in triplicate, and four fields of each plate were recorded. (c) HMEC-1 cells (2×10^4) were seeded into the upper chamber of the transwell and the lower chamber was filled with 0.8 mL low-glucose Dulbecco's modified Eagle's medium supplemented with vascular endothelial growth factor (10 ng/mL) to induce chemotaxis. After 24 h of incubation at 37°C, 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. (d) For fluorescence-activated cell sorter analysis, cells were incubated in serum-free media for 24 h, and annexin V/Cy5 and propidium iodide were added for incubation for 30 min. All experiments were repeated three times with similar results.

of endothelial proliferation, motility, capillary formation, and stability induced by VEGF.[24,25] We hypothesize that inhibiting angiogenesis by targeting RhoC is not exclusively dependant on the VEGF signaling pathway. In our neovascularization model *in vivo*, the PT67 cell line was inoculated subcutaneously as a constant source of retrovirus, whereas matrigel mixed with the same concentration of VEGF was inoculated subcutaneously for specific investigation of VEGF-driven angiogenesis. Our results based on this neovascularization model *in vivo* show that knockdown of RhoC inhibits angiogenesis induced by VEGF, suggesting that RhoC is the downstream regulator of VEGF in endothelial cells and is essential for angiogenesis induced by VEGF, notwithstanding that RhoC regulates the expression of VEGF in tumor cells.

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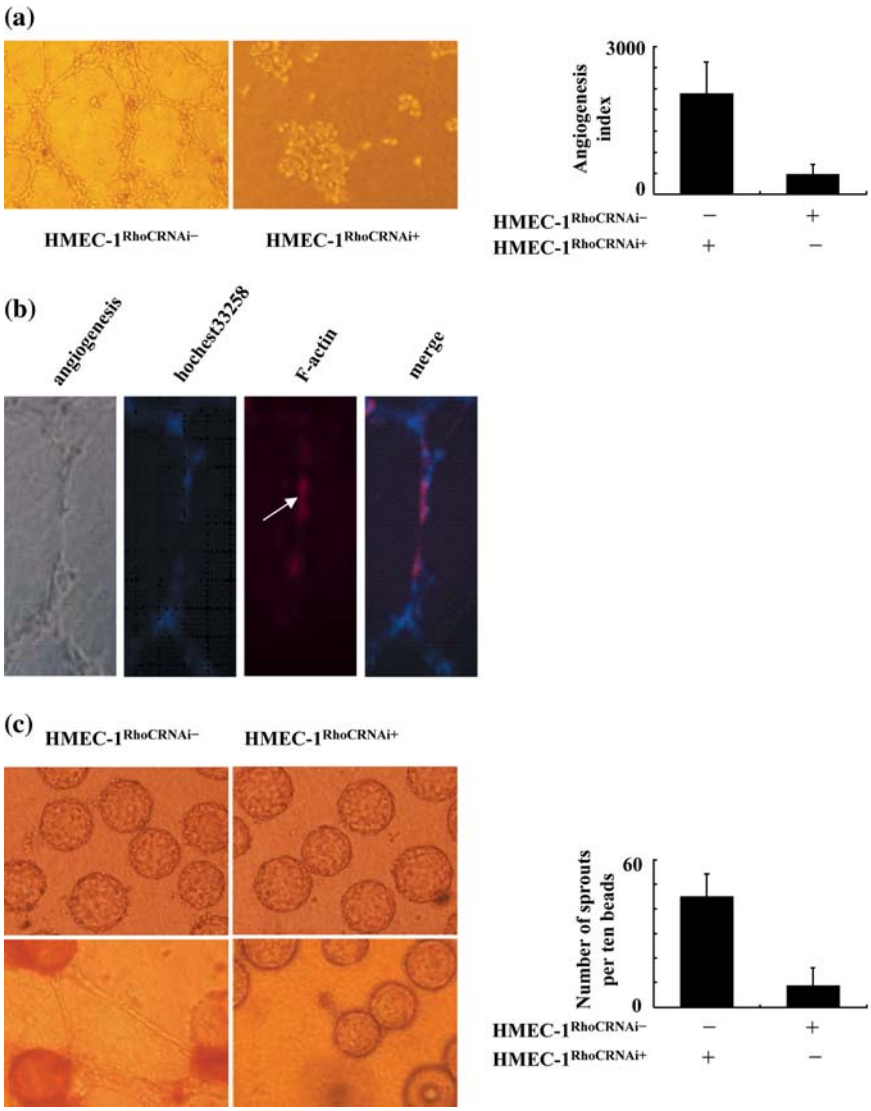

Fig. 4. Knockdown of RhoC inhibits angiogenesis *in vitro*. (a) HMEC-1 cells were seeded on matrigel and incubated for 18 h. The network formation was observed. The average number of network projections in 10 × 40 fields was counted for three independent experiments in each group, and the data were obtained using the following formula: angiogenesis = $a \times b$, where a = the number of branch points per ×40 field and b = the total number branches per point. (b) F-actin filaments were visualized in cells using rhodamine-conjugated phalloidin (white arrow). (c) HMEC-1 cells were mixed and incubated with Cytodex-3 microcarriers, then HMEC-1 cells, adherent to microcarriers, were mixed with matrigel and cultured for 1 week. The sprouting of HMEC-1 cells was photographed and the number of sprouts per bead determined, where a sprout was defined as a vessel of length equal to the diameter of a bead. For statistical analysis, the number of sprouts/10 beans was counted and 100 beads were assessed for each condition. Experiments were repeated three times with similar results. The results are shown as the mean ± SD. Columns, mean (n = 3); bar, SD.

We showed that RhoC is essential for angiogenesis induced by VEGF, and RhoC is the downstream regulator of VEGF in endothelial cells. However, there was no evidence indicating the direct regulation of VEGF to RhoC. In fact, the function of RhoC protein in regulating the actin cytoskeleton is universal, which implies that RhoC participation in angiogenesis is not dependant on VEGF. Here, we showed that knockdown of RhoC is associated with the inhibition of invasion and migration but not apoptosis of HMEC-1 cells, consistent with the critical role of RhoC in the control of cellular motility.[3] Furthermore, our results show that inhibition of RhoC results in inhibition of HMEC-1 organization through inhibiting reorganization of F-actin filaments. As expected, knockdown of RhoC inhibited tube formation of HMEC-1 in three-dimensional matrigel. Together, these results demonstrate that knockdown of RhoC inhibits angiogenesis through inhibiting endothelial cell migration and organization.

Acknowledgments

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