Available online at www.sciencedirect.com
SCIENCE DIRECT
Biochemical and Biophysical Research Communications 346 (2006) 74–82
BBRC
www.elsevier.com/locate/ybbrc

RhoC is essential for TGF-β1-induced invasive capacity of rat ascites hepatoma cells

M. Mukai [a], H. Endo [a], T. Iwasaki [b], M. Tatsuta [c], A. Togawa [d], H. Nakamura [a], M. Inoue [a,*]

[a] Department of Biochemistry, Osaka Medical Center for Cancer and Cardiovascular Diseases, Osaka 537-8511, Japan
[b] Department of Respiratory Surgery, Osaka Prefectural Medical Center for Respiratory and Allergic Diseases, Osaka 583-8588, Japan
[c] Department of Gastrointestinal Oncology, Osaka Medical Center for Cancer and Cardiovascular Diseases, Osaka 537-8511, Japan
[d] RIKEN Center for Developmental Biology, Laboratory for Stem Cell Research, Kobe 650-0047, Japan

Received 11 May 2006
Available online 19 May 2006

Abstract

Transforming growth factor-β1 (TGF-β1) is a multifunctional growth factor that plays a role in cell proliferation, differentiation, extracellular matrix production, apoptosis, and cell motility. We show here that TGF-β1 increased the invasiveness of MM1 cells, which are a highly invasive clone of rat ascites hepatoma cells. Both mRNA and protein levels of RhoC but not RhoA in TGF-β1-treated MM1 cells increased. In parallel with this increase in expression, RhoC activity was induced by TGF-β1 treatment. When RhoC was overexpressed in MM1 cells, the invasive capacity increased. The RhoC-overexpressing cells formed more nodules than did mock cells when injected into rat peritoneum. Furthermore, when RhoC expression was reduced by transfection with shRNA/RhoC, the invasiveness of MM1 cells decreased with concomitant suppression of RhoC expression. Thus, the induced expression of RhoC by TGF-β1 in MM1 cells plays a critical role in TGF-β1-induced cell migration.

Keywords: RhoA; RhoC; TGF-β1; Invasion

TGF-β is a multifunctional peptide regulator that controls cell proliferation, differentiation, angiogenesis, extracellular matrix deposition, and tumor invasion and metastasis [1]. TGF-β is produced by a variety of tumor cells, as well as by platelets. Increased expression of TGF-β1 has been found in many human cancers relative to non-transformed cells, and the level of TGF-β1 is correlated with enhanced invasion and metastasis of the tumor [2]. We previously reported that TGF-β1 induces invasive capacity in W1 cells, a poorly invasive clone of rat ascites hepatoma AH130 cells [3]. TGF-β1 up-regulates transcription of various genes that potentiate the invasive capacity of tumor cells, such as urokinases in human breast cancer cells [4], αvβ3 integrin in human glioma cells [5], α3β1 integrin in human hepatoma cells [6], and CXCR4 in human melanoma cells [7].

TGF-β1 reportedly affects cell morphology, which involves dynamic cytoskeletal reorganization and profoundly relates to cell migration, in a number of different cell types, such as mink lung epithelial Mv1Lu cells [8], Swiss 3T3 cells [9], and human epidermoid carcinoma KB cells [10]. This effect is presumably dependent on changes in the cytoskeleton caused by altered expression of cytoskeletal elements, such as smooth muscle actin [11], vimentin, and tubulin [12], as well as assembly of actin filaments into stress fibers [10].

The Rho GTPases are the pivotal regulators of the actin filament system [13,14]. Overexpression of RhoA, one of these RhoGTPase is commonly found in malignant tumors [15], and manipulation of the level of RhoA expressed in various transformed cell lines has been found to affect their aggressiveness [16–18]. We have previously revealed that

* Corresponding author. Fax: +81 6 6973 5691.
E-mail address: inoue-ma2@mc.pref.osaka.jp (M. Inoue).

0006-291X/$ - see front matter © 2006 Elsevier Inc. All rights reserved.
doi:10.1016/j.bbrc.2006.05.068

MM1 cells, a highly invasive clone of rat ascites hepatoma AH130 cells, show invasive capacity in a Rho-mediated manner [19,20]. RhoC, another member of RhoGTPases, has a high homology with RhoA. A connection between cancer metastasis and RhoC has been suggested in various reports. High expression levels of RhoC were reported to correlate with clinical cancer metastasis [21–24]. In animal studies, RhoC is associated with experimental pulmonary metastasis of melanoma cell lines [25]. In addition, pulmonary metastasis of mammary adenocarcinoma in the Polyomavirus Middle T transgenic and RhoC$^{-/-}$ mouse was drastically inhibited [26]. RhoC is also found to be associated with cell line invasiveness in vitro [22,24].

TGF-β1 rapidly activates RhoA in an epithelial, nontransformed mouse mammary cell line (NmuMG), a mink lung cell line (Mv1Lu), a pancreatic tumor cell line (BxPc3), and primary mouse keratinocytes [27]. Meanwhile, Edlund et al. [28] have reported that prolonged treatment of PC-3U cells with TGF-β1 results in the formation of stress fibers as well as cortical actin filaments and that this requires a cooperation between Smad and Rho GTPase signaling pathways. Taken together, it is reasonable to have a hypothesis that TGF-β1-induced invasiveness of cancer cells is relevant to Rho GTPase activity. Here, we investigated the distinct role of RhoA and RhoC in TGF-β1-induced invasiveness of MM1 cells.

Materials and methods

Reagents. TGF-β1 and a rabbit anti-TGF-β1 polyclonal antibody were obtained from R&D Systems (Minneapolis, MN). Anti-RhoA antibody was purchased from Santa Cruz Biotechnology (Santa Cruz, CA). Polyclonal anti-RhoC antibody was generated by immunizing rabbits with C-terminal region peptide (GLVQVRKNKRRRGCPIL) of RhoC [29]. 1-Oleoyl-*sn*-lysophosphatidic acid (LPA) was purchased from Sigma Chemical Co. (St. Louis, MO) and dissolved in PBS supplemented with 0.1% BSA.

Cell, cell culture, and transfection. The MM1 cells were cultured in suspension in DMEM supplemented with 10% FBS. Cells were cultured at 37 °C in a humidified atmosphere of 5% CO_2 in air. The morphology of MM1 cells was observed under a phase-contrast microscope (Olympus IX70, Tokyo, Japan). A retrovirus vector containing human RhoC cDNA, pMX-IRES-GFP/RhoC, was transfected into plat-E packaging cells using FuGene (Roche, Indianapolis, IN). Tissue culture supernatant was harvested 48 h later and added to MM1 cells in the presence of 8 μg/ml polybrene. Infected cells were selected by limited dilution. An expression vector containing a myc-tagged human RhoA cDNA, pCDNA3/myc-RhoA, was transfected into HEK293 cells using FuGene (Roche). After 48 h, the cells were lysed with sample buffer (Wako, Osaka, Japan), and Western blots were performed.

Transcellular migration (in vitro invasion) assay. The assay procedure to measure the in vitro invasive capacity of tumor cells was essentially the same as previously described [30]. MM1 cells (2×10^5 cells) were seeded over a rat mesothelial cell monolayer (MCL) and cultured in DMEM supplemented with 10% FBS. After incubation, the supernatant was removed, and the resultant monolayer was fixed in situ with 10% formalin. The number of penetrated single tumor cells and tumor cell colonies (collectively called invasion foci) was counted under a phase-contrast microscope.

Semi-quantitative RT-PCR. Total RNA from MM1 cells cultured with or without TGF-β1 was extracted with Trizol (Invitrogen, Carlsbad, CA). The same amount of RNA was reverse transcribed to obtain cDNA using Ready-To-Go beads (Amersham Bioscience, Tokyo, Japan). Semi-quantitative PCRs were performed with the following conditions: an initial step of 5 min at 94 °C, differential cycles of 1 min at 94 °C, 1 min at 58 °C, and 30 s at 72 °C, followed by a step at 72 °C for 10 min. The cycle numbers for RhoA, RhoC, and β-actin were 21, 25, and 21, respectively. The levels of Rho-A and Rho-C expression were standardized by the levels of β-actin. The primer sequences were as follows: rat Rho-A, 5′-AAAGTC GGGGTGCCTCA-3′ and 5′-GAGGGCGTTAGAGCAGTGTC-3′; rat Rho-C, 5′-GCCTACAGGTCCGGAAGAAT-3′ and 5′-GCACCAACCT AGTTCCCAGA-3′; and rat β-actin, 5′-CCTGAGGAGCACCCTGTG-3′ and 5′-AACACAGCCTGGATGGCTAC-3′.

Western blotting. Western blot analysis was performed by essentially the same method as previously described [19]. Briefly, proteins in an SDS–polyacrylamide gel were transferred electrophoretically to a PVDF membrane. The membrane was blocked with 5% milk or 1% BSA solution in T-TBS (Tris–HCl-buffered saline supplemented with 0.5% Tween 20), and incubated with a primary antibody in T-TBS overnight. After washing with T-TBS, the membrane was allowed to react with a suitable secondary antibody conjugated with horseradish peroxidase. After extensive washing, signals were detected using an ECL-detecting reagent (Amersham-Pharmacia Biotech, Uppsala, Sweden). Protein concentration was determined by Protein Assay (Bio-Rad, Hercules, CA).

RhoA and RhoC activity assay. MM1 cells were washed twice with DMEM and incubated in fresh DMEM without serum for 3 h; 25 μM LPA was added and the culture was centrifuged. The cell pellet was lysed in RIPA buffer (50 mM Tris, pH 7.5, 1% Triton X-100, 0.5% sodium deoxycholate, 0.1% SDS, 500 mM NaCl, 10 μg/ml each of leupeptin and aprotinin, and 1 mM PMSF). Cell lysates were clarified by centrifugation at 13,000*g* at 4 °C for 10 min and were incubated for 45 min with Rhotekin Rho Binding Domain-agarose (Upstate, Lake Placid, NY). The agarose beads were washed three times with buffer B (Tris buffer containing 1% Triton X-100, 150 mM NaCl, 10 mM $MgCl_2$, 10 μg/ml each of leupeptin and aprotinin, and 0.1 mM PMSF). Rho bound to the beads, which is a GTP-bound form of Rho proteins, was detected by Western blotting using anti-RhoA or RhoC antibodies.

In vivo invasion assay. MM1 cells (2×10^7 cells), stably expressing RhoC or empty vector only (mock), were injected into the peritoneal cavity of the syngeneic male Donryu rats (150-g body weight). After 14 days, all animals were sacrificed and the number of tumor nodules in the peritoneal cavity was counted.

Gene silencing of the rat RhoA and RhoC genes. Gene silencing was performed using shRNA with the pSuperRetro plasmid (Oligoengine, Seattle, WA). In brief, a duplex oligonucleotide including the targeting sequences of the rat Rho-A (5′-CAAGAAGGATCTTCGGAAT-3′) or Rho-C (5′-GGACCTGAGGCAAGATGAG-3′) genes was subcloned into a pSuperRetro that had been digested with *Bgl*II and *Hin*dIII. As a control, an empty pSuperRetro plasmid was used. All clones were verified by sequencing. The plasmids were transfected into plat-E packaging cells using FuGene 6, and 48 h after transfection, cell culture medium supplemented with 8 μg/ml polybrene was used to infect MM1 cells. The infected cells were selected with puromycin (8 μg/ml), and clones were isolated by limiting dilution.

Statistical analysis. Statistical analysis was performed with GraphPad Prism 4 (GraphPad Software, San Diego, CA). The statistical significance of the results was tested with an unpaired-*t* test. A value of $p < 0.05$ was considered to be statistically significant.

Results

TGF-β1 induces invasiveness with morphological changes of MM1 cells

TGF-β1 treatment increased the invasive capacity of MM1 cells dose dependently, reaching a maximum 2.5-fold increase after 24-h treatment with TGF-β1, 10 ng/ml (Fig. 1A). The invasive capacity increased over time up to 48 h after treatment with 10 ng/ml TGF-β1 (Fig. 1B). The

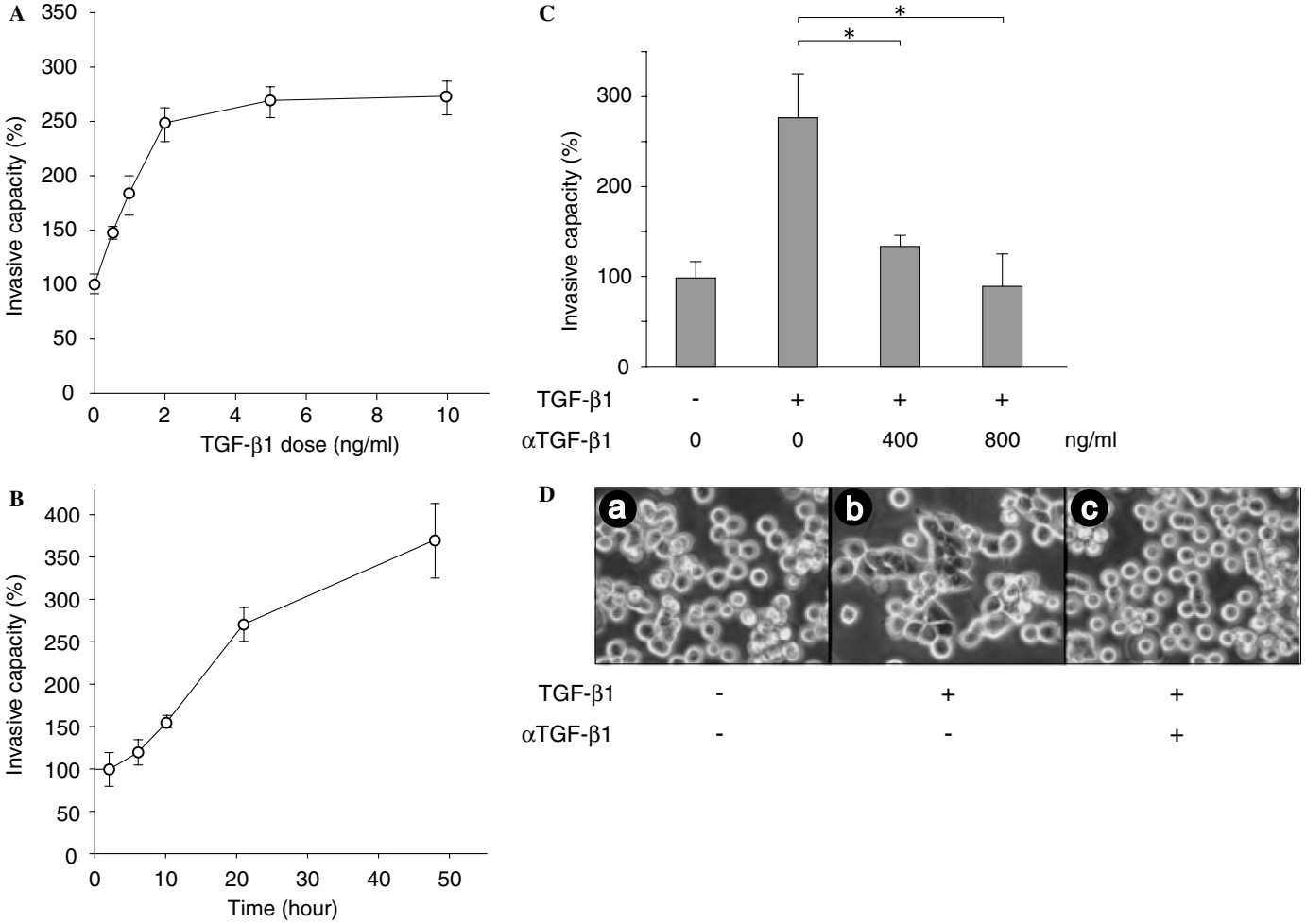

Fig. 1. TGF-β1-induced invasiveness in MM1 cells with morphological changes. (A) MM1 cells were incubated with indicated doses of TGF-β1 and the invasive capacity was assessed. Relative invasive capacity to that of non-treated cells is shown. (B) MM1 cells were incubated with 10 ng/ml TGF-β1 for the indicated periods and invasive capacity was assessed. (C) TGF-β1, 10 ng/ml, was incubated with the indicated concentrations of the anti-TGF-β1 antibody for 1 h at room temperature; then MM1 cells were added and incubated at 37 °C for 24 h, and the invasive capacity was assessed. Data are shown as mean values ±SD; *, $p < 0.05$. (D) Phase-contrast images of the cells are shown. Note that the morphological changes of MM1 cells induced by TGF-β1 (b) were abolished to the levels of non-treated cells (a) by pre-incubation with anti-TGF-β1 antibody, 800 ng/ml (c).

TGF-β1-induced invasive capacity was inhibited dose dependently by addition of anti-TGF-β1 antibody (Fig. 1C). On the other hand, PDGF or EGF did not increase invasive capacity (data not shown). Next, the morphological changes of TGF-β1-treated MM1 cells were examined. Previously, we reported that MM1 cells, treated with 1-oleoyl lysophosphatidic acid (LPA) on a fibronectin (FN)-coated dish, exhibited a unidirectional extended fusiform shape that was concomitant with invasion of MM1 cells [20]. MM1 cells treated with TGF-β1 showed strong adhesion to dishes and a fusiform shape (Fig. 1D). Anti-TGF-β1 antibody abrogated the TGF-β1-induced morphological changes. These results indicate that TGF-β1 induces invasive capacity and the concomitant morphological changes in MM1 cells.

TGF-β1 increases RhoC mRNA and protein expression

We previously revealed that the invasion of MM1 cells is tightly connected to RhoA activation [20]. We studied the role of RhoA and RhoC in the TGF-β1-induced invasive capacity of these cells. First, we investigated the expression levels of RhoA and RhoC mRNA in MM1 cells with TGF-β1 treatment using semi-quantative PCR. As shown in Fig. 2A and B, 24-h treatment of MM1 cells with TGF-β1 dose dependently increased RhoC mRNA levels but did not change RhoA levels. The levels of RhoC mRNA increased over time from 8 h after treatment with 10 ng/ml TGF-β1 (Fig. 2C and D). By contrast, RhoA mRNA levels did not change throughout the experimental period. Next, the expression of RhoA and RhoC was examined at the protein level by Western blot analysis. Prior to the experiment, the specificity of the antibodies was verified, using HEK293 cells overexpressing myc-tagged RhoA. Anti-myc antibody detected only the exogenous myc/RhoA, while anti-RhoA antibody detected two bands corresponding to myc/RhoA and endogenous RhoA (Fig. 2E). When anti-RhoC antibody was used, myc/RhoA was not detected. Instead, a band corresponding to the size

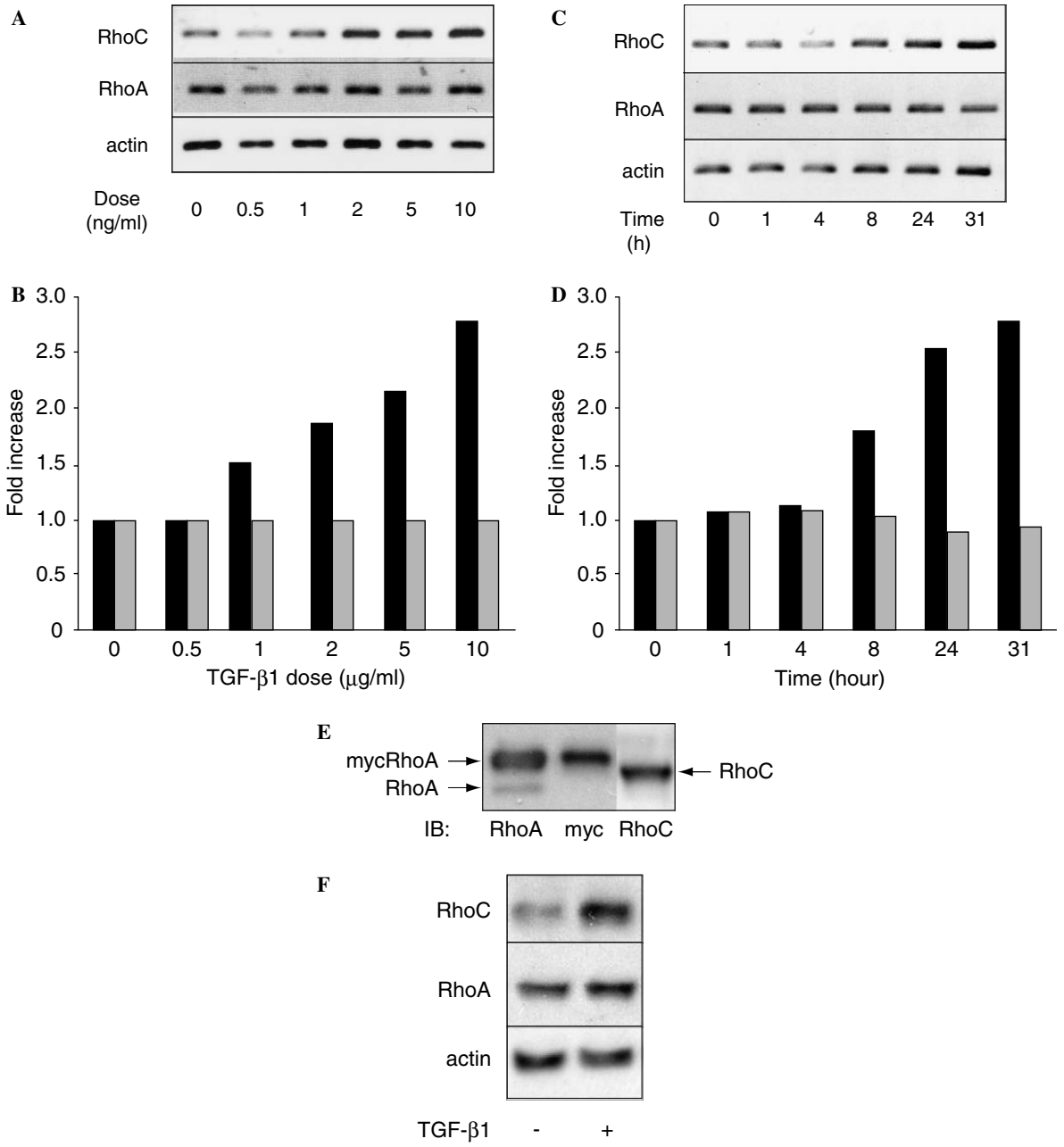

Fig. 2. TGF-β1 increased RhoC mRNA and protein expression. (A–D) Semi-quantitative RT-PCR of RhoC and RhoA messenger RNA from MM1 cells cultured for 24 h with various doses of TGF-β1 (A,B) or cultured for various periods with 10 ng/ml TGF-β1 (C,D). The results of densitometric analysis are corrected against actin values and shown as the relative increase over the value of non-treated cells (B,D). The black bar and the grey bar represent the results of RhoC and RhoA, respectively. The experiments were performed three times, and representative data are shown. (E) Western blotting from myc-RhoA-transfected HEK293 cells. Three strips of the membrane were cut out, and each strip was immunoblotted using the indicated antibodies. The gene products corresponding to the bands are indicated by arrows. (F) Western blotting of the cell lysate from TGF-β1 treatment, 10 ng/ml for 24 h, and from non-treated MM1 cells. The antibodies used are indicated.

of RhoC was detected at the location between myc/RhoA and endogenous RhoA. Thus, anti-RhoC antibody was shown not to cross-react with RhoA and vice versa. Using the antibodies, we examined the change in protein levels of RhoA and RhoC after TGF-β1 treatment. RhoC levels, but not RhoA levels, increased in MM1 cells treated with 10 ng/ml TGF-β1 after 24 h of treatment, consistent with the results from measuring mRNA levels (Fig. 2F).

TGF-β1 increased the activity of RhoC but not RhoA

Because TGF-β1 induced the expression of RhoC but not RhoA at both the mRNA and protein levels, the activation status of RhoA and RhoC by TGF-β1 was examined. Previously, we showed that LPA activates RhoA in MM1 cells [20]. In the cell lysates (Fig. 3, left panels), TGF-β1 increased expression levels of RhoC but not of

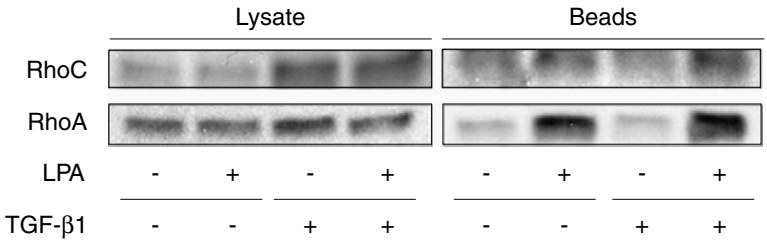

Fig. 3. The activity of RhoC but not RhoA was increased by TGF-β1. MM1 cells were pretreated for 24 h with or without TGF-β1 and then cultured with FCS-free medium for 2 h. Ten minutes after the addition of LPA, cells were lysed and subjected to the Rho-binding assay. The results from the cell lysate and the beads are shown in the left and right panels, respectively. The antibodies used are indicated.

RhoA, consistent with the above data, while LPA did not change the expression levels of either. In terms of the activation levels of the Rho GTPases, assessed by pull-down assay using the GST-fused Rho-binding domain (RBD) of Rhotekin (Fig. 3, right panels), LPA activated both RhoA and RhoC. In the absence of LPA, neither RhoA nor RhoC was activated by TGF-β1; however, RhoC but not RhoA was activated by TGF-β1 in the presence of LPA. It should be noted that treatment with TGF-β1 alone did not increase the activation levels of RhoC in spite of the increase in RhoC protein levels. Taken together, these data show that TGF-β1 augmented the LPA-induced activation of RhoC by increasing the levels of RhoC protein.

Overexpression of RhoC increased invasiveness of MM1 cells

To examine whether the increased level of RhoC expression is sufficient to induce invasion, human RhoC cDNA was transfected into MM1 cells. Several stable clones were isolated by limiting dilution, and three clones with different RhoC overexpression levels—CO-3 (low), CO-12 (intermediate), and CO-6 (high)—were subjected to further studies. First, RhoC expression levels in the clones were estimated by the intensity of EGFP fluorescence (Fig. 4A). Because EGFP was translated through an internal ribosomal entry site in the RhoC expression vector, the levels of RhoC expression could be estimated by the intensity of EGFP fluorescence. Then, the expression levels of RhoC were confirmed by Western blotting using anti-RhoC antibody (Fig. 4B). Invasive capacity in vitro was increased only in the cells with high expression but not in the low- and intermediate-expression clones (Fig. 4C). Next, the clone with high expression of RhoC (CO-6) was intraperitoneally transplanted in rats. As shown in Fig. 5A, 14 days after injection, more tumor nodules were formed in rats injected with cells overexpressing RhoC than in rats injected with mock-transfected cells. No significant difference was observed in cell growth in vitro between the clones (Fig. 5B). These results indicate that invasiveness was

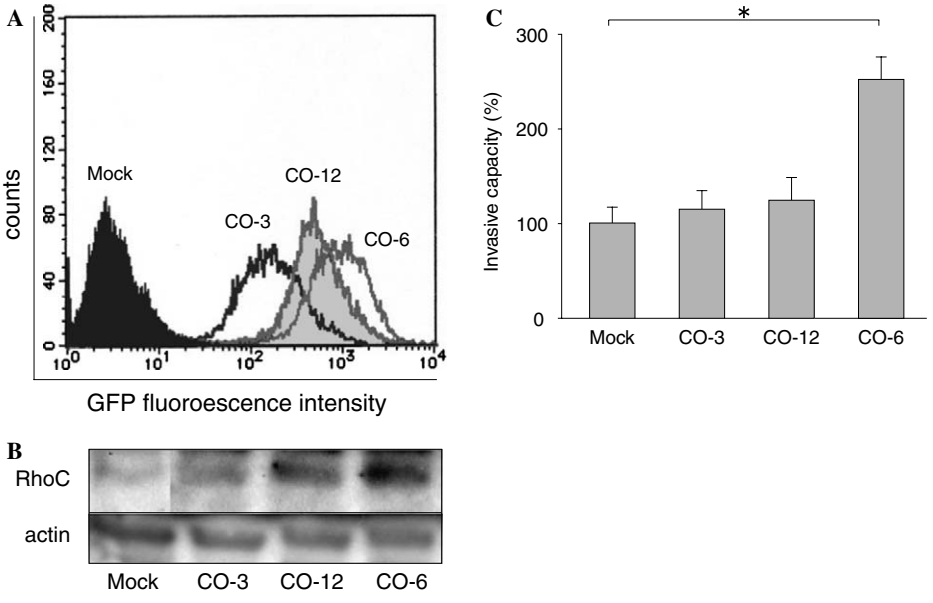

Fig. 4. Overexpression of RhoC increased invasive capacity both in vitro and in vivo. (A) Fluorescence of EGFP from three clones of RhoC overexpressing cells was analyzed by flow cytometry. An open area edged with black line, CO-3; an open area edged with grey line, CO-6; a grey area edged with black line, CO-12. (B) Western blotting of the clones using RhoC antibody. (C) Invasive capacity of the RhoC-overexpressing clones. Data are shown as percent increase of invasive capacity over that of the empty-vector-transfected clone (Mock). Columns, mean of three results; bars, SD; *, $p < 0.05$.

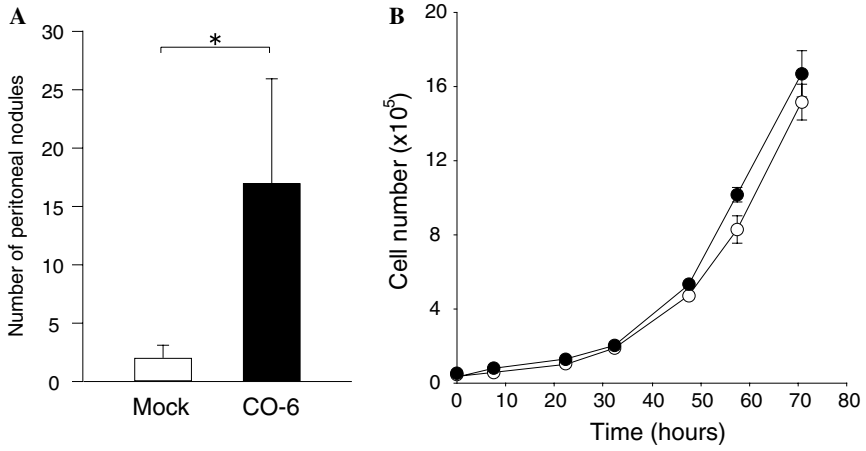

Fig. 5. (A) Number of nodules formed in the rat peritoneum 14 days after injection of the empty vector-transfected clone (Mock) and the CO-6 clone. Columns, mean of three results; bars, SD; *, $p < 0.05$. (B) Growth curve of Mock (filled circle) and CO-6 clone (open circle) in vitro.

increased in cells overexpressing RhoC in vitro as well as in vivo.

Inhibition of RhoC expression decreased invasiveness

Next, we examined whether RhoC is necessary for TGF-β1-induced invasion. We applied RNA small hairpin (shRNA) techniques to decrease rat RhoA and RhoC gene expression. Two clones were isolated from the cells transfected with RhoC-shRNA vector; CI-2 and CI-8. Protein levels of RhoC but not RhoA were remarkably decreased in these two clones (Fig. 6A). The invasive capacity of CI-2 and CI-8 with TGF-β1 treatment was examined (Fig. 6B). Decreased invasive capacity was observed in both CI-2 and CI-8 cells compared with mock transfected cells. TGF-β1-induced invasive capacity was also decreased in these cells. Invasive capacity was especially almost abrogated in CI-8 cells, which exhibited lower expression levels of RhoC than did CI-2 cells. Next, two clones were isolated from the mixture of RhoA–shRNA vector-transfected

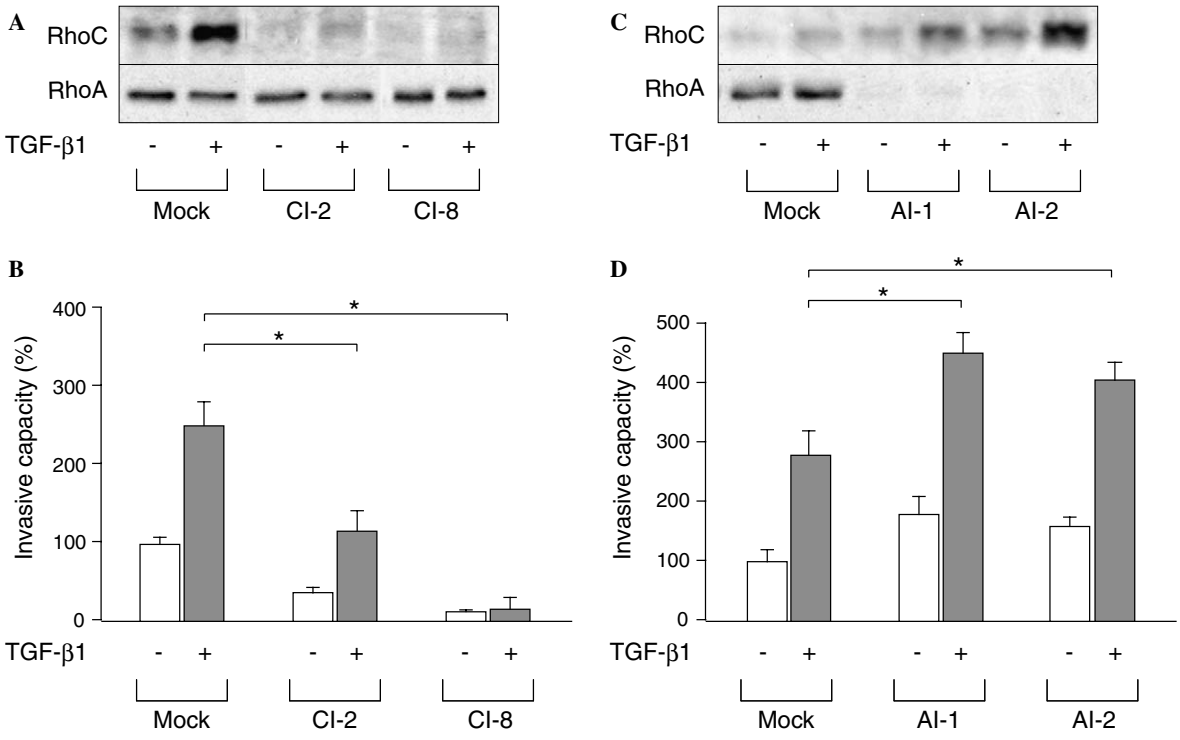

Fig. 6. Inhibition of RhoC expression decreased invasive capacity. (A,C) Western blotting of the clones transfected with shRNA of RhoC; CI-2, CI-8 (A), and RhoA; AI-1, AI-2 (C), with or without TGF-β1 treatment. The antibodies used are indicated. (B,D) Invasive capacity of the clones transfected with shRNA of RhoC (B) and RhoA (D). Invasive capacity was measured with or without TGF-β1 treatment. Data are shown as percent increase of invasive capacity over that of the empty-vector-transfected clone (Mock). Columns, mean of three results; bars, SD; *, $p < 0.05$.

cells, AI-1 and AI-2. Protein levels of RhoA but not RhoC were remarkably decreased in these two clones (Fig. 6C). Intriguingly, when RhoA expression was suppressed, RhoC expression was reciprocally upregulated and more notable when stimulated by TGF-β1.

The invasive capacity of AI-1 and AI-2 with TGF-β1 treatment was examined (Fig. 6D). Increased invasive capacity was observed in both AI-1 and AI-2 cells compared with mock-transfected cells, and it was more distinct in cells stimulated with TGF-β1. Thus, RhoC was indispensable for TGF-β1-induced invasive capacity, while absence of RhoA rather increased invasive capacity, probably because of compensatory induction in RhoC expression.

Discussion

In the present study, we have shown that TGF-β1 induces RhoC mRNA and protein expression, in parallel with enhanced invasiveness of MM1 cells, a clone derived from rat ascites hepatoma cells. Although there are several reports showing that TGF-β1 activates RhoA [27,28,31,32], this is the first report, to our knowledge, showing that TGF-β1-induced tumor invasiveness is exerted with an increase in RhoC expression levels.

The RhoC gene is highly homologous with that of RhoA. Both RhoA and RhoC interact with the downstream effector Rho kinase (ROCK), and RhoC exhibits a higher affinity for ROCK than does RhoA [33]; based on this stronger affinity, one may predict a prominent role for RhoC in cancer cell invasion. Until recently, the discrete signals of RhoA and RhoC have not been clarified. Previous studies, especially studies on Rho protein function, have used dominant-negative and constitutive-active approaches, alongside biochemical ablation by *Clostridium difficile* toxin or *Clostridium botulinum* C3 exoenzyme treatment, all of which cannot distinguish individual Rho isoforms.

Recently, several investigators using new technologies reported a functional difference between RhoA and RhoC. Using the RNA interference technique, Simpson et al. [34] reported that RhoA impedes and RhoC stimulates LPA-induced invasion of SUM-159 breast cancer cells. They also reported that in MCF-7 cells, inhibition of RhoA gene expression resulted in a substantial increase in migration toward NIH-3T3-conditioned media. Wang et al. [35] used forced expression of the fusion proteins of p190 RhoGap and the c-terminus sequence of Rho isoforms to inactivate individual Rho proteins. They showed that invasion of A375-M human melanoma cells was suppressed by RhoC-targeted RhoGAP, but not by RhoGAP that was RhoA targeted. These reports are consistent with our current results showing that TGF-β1-induced invasion of MM1 cells was suppressed by RhoC knockdown, while the invasiveness was rather enhanced by RhoA knockdown. On the other hand, Pille et al. [36] reported that suppression of both RhoA and RhoC expression by siRNA inhibited migration of MDA-MB-231 breast cancer cells attracted by bFGF. The discrepancy might result from the dramatic suppression of proliferation by siRNA of Rho GTPases in the experiment.

The contribution of RhoC to cell migration might also be cell-type specific. In our previous study using W1 cells, derived from a poorly invasive clone of AH130, we showed that their invasive capacity increased with TGF-β1 treatment [3]. Although both mRNA and protein levels of RhoC were induced by TGF-β1 in W1 cells, overexpression of RhoC in W1 cells did not increase the invasive capacity (data not shown). In W1 cells, upregulation of RhoC alone is not enough to induce invasive capacity. Cooperation of a set of genes might be required to potentiate invasive capacity by TGF-β1 in W1 cells.

Our study revealed that induction of RhoC mRNA is essential for demonstration of the invasive capacity induced by TGF-β1. Because cycloheximide did not inhibit TGF-β1-induced RhoC mRNA levels (data not shown), it is likely that transcriptional machinery, directly downstream of TGF-β1, is responsible for the increase of RhoC mRNA, although a regulatory mechanism of the RhoC promoter region is thus far poorly understood. The overexpression of RhoC commonly observed in malignant tumor samples [21–24] might be a consequence of growth factor stimulation. RhoC expression under stimulation of other growth factors, which induce invasive capacity, would be worth further studies.

Another intriguing finding is the responsive upregulation of RhoC protein levels by RhoA inhibition. As shown in Fig. 6, we revealed that expression of RhoC increased in response to the decrease of RhoA expression. Simpson et al. [34] reported that in the absence of RhoA expression, LPA treatment significantly increased Rac1 activity. These results suggest that enhancement of invasion by RNA interference of RhoA in this study might result from the compensatory increase of other Rho GTPases, including RhoC, but not from the suppressive role of RhoA per se.

Muraoka et al. [37] reported that blockade of TGF-β signaling with a soluble Fc:TGF-β type II receptor fusion protein inhibited the formation of distant metastases in experimental models of breast cancer. Because of the critical role of RhoC in the TGF-β1-induced invasive capacity of MM1 cells, the inhibition of RhoC activity might be a possible approach to the treatment of invasion and metastasis of cancer cells. Various methods of blocking Rho-GTPases have been applied to inhibit invasion and metastasis of cancer cells, such as GGTI [38] and HMG-CoA reductase inhibitor [39], although none were specific to RhoA or RhoC. To treat a cancer in which TGF-β1 stimulation or high expression of RhoC is the major cause of invasive capacity, targeting RhoC alone might be a better approach than targeting a broad range of Rho GTPases.

Acknowledgments

This work has been supported by a Grant-in-Aid from the Ministry of Health and Welfare for the Second-Term

Comprehensive 10-Year Strategy for Cancer Control, Japan. We thank Y. Mawatari and A. Fukano for technical assistance.

References

[1] G.C. Blobe, W.P. Schiemann, H.F. Lodish, Role of transforming growth factor beta in human disease, N. Engl. J. Med. 342 (2000) 1350–1358.
[2] R. Derynck, R.J. Akhurst, A. Balmain, TGF-β signaling in tumor suppression and cancer progression, Nat. Genet. 29 (2001) 117–129.
[3] M. Mukai, K. Shinkai, K. Komatsu, H. Akedo, Potentiation of invasive capacity of rat ascites hepatoma cells by transforming growth factor-β, Jpn. J. Cancer Res. 80 (1989) 107–110.
[4] A.R. Farina, A. Coppa, A. Tiberio, A. Tacconelli, A. Turco, G. Colletta, A. Gulino, A.R. Mackay, Transforming growth factor-beta1 enhances the invasiveness of human MDA-MB-231 breast cancer cells by up-regulating urokinase activity, Int. J. Cancer 75 (1998) 721–730.
[5] M. Platten, W. Wick, C. Wild-Bode, S. Aulwurm, J. Dichgans, M. Weller, Transforming growth factors β(1) (TGF-β(1)) and TGF-β(2) promote glioma cell migration via Up-regulation of α(V)β(3) integrin expression, Biochem. Biophys. Res. Commun. 268 (2000) 607–611.
[6] G. Giannelli, E. Fransvea, F. Marinosci, C. Bergamini, S. Colucci, O. Schiraldi, S. Antonaci, Transforming growth factor-β1 triggers hepatocellular carcinoma invasiveness via α3β1 integrin, Am. J. Pathol. 161 (2002) 183–193.
[7] R.A. Bartolome, B.G. Galvez, N. Longo, F. Baleux, G.N. Van Muijen, P. Sanchez-Mateos, A.G. Arroyo, J. Teixido, Stromal cell-derived factor-1α promotes melanoma cell invasion across basement membranes involving stimulation of membrane-type 1 matrix metalloproteinase and Rho GTPase activities, Cancer Res. 64 (2004) 2534–2543.
[8] B. Like, J. Massague, The antiproliferative effect of type beta transforming growth factor occurs at a level distal from receptors for growth-activating factors, J. Biol. Chem. 261 (1986) 13426–13429.
[9] K.M. Lee, J. Park, J.H. Kim, S.W. Yie, G.T. Chun, P.H. Kim, E.Y. Choi, Reorganization of myosin and focal adhesion proteins in Swiss 3T3 fibroblasts induced by transforming growth factor β, Cell Biol. Int. 23 (1999) 507–517.
[10] S. Koyasu, T. Kadowaki, E. Nishida, K. Tobe, E. Abe, M. Kasuga, H. Sakai, I. Yahara, Alteration in growth, cell morphology, and cytoskeletal structures of KB cells induced by epidermal growth factor and transforming growth factor-β, Exp. Cell Res. 176 (1988) 107–116.
[11] Y. Nakajima, T. Yamagishi, K. Yoshimura, M. Nomura, H. Nakamura, Antisense oligodeoxynucleotide complementary to smooth muscle alpha-actin inhibits endothelial-mesenchymal transformation during chick cardiogenesis, Dev. Dyn. 216 (1999) 489–498.
[12] A. Lomri, P.J. Marie, Effects of transforming growth factor type beta on expression of cytoskeletal proteins in endosteal mouse osteoblastic cells, Bone 11 (1990) 445–451.
[13] A. Hall, Rho GTPases and the actin cytoskeleton, Science 279 (1998) 509–514.
[14] P. Aspenstrom, Effectors for the Rho GTPases, Curr. Opin. Cell Biol. 11 (1999) 95–102.
[15] G. Fritz, I. Just, B. Kaina, Rho GTPases are over-expressed in human tumors, Int. J. Cancer 81 (1999) 682–687.
[16] L. del Peso, R. Hernandez-Alcoceba, N. Embade, A. Carnero, P. Esteve, C. Paje, J.C. Lacal, Rho proteins induce metastatic properties in vivo, Oncogene 15 (1997) 3047–3057.
[17] J.C. Stam, F. Michiels, R.A. van der Kammen, W.H. Moolenaar, J.G. Collard, Invasion of T-lymphoma cells: cooperation between Rho family GTPases and lysophospholipid receptor signaling, Embo J. 17 (1998) 4066–4074.
[18] K. Yoshioka, S. Nakamori, K. Itoh, Overexpression of small GTP-binding protein RhoA promotes invasion of tumor cells, Cancer Res. 59 (1999) 2004–2010.
[19] F. Imamura, K. Shinkai, M. Mukai, K. Yoshioka, R. Komagome, T. Iwasaki, H. Akedo, Rho-mediated protein tyrosine phosphorylation in lysophosphatidic-acid-induced tumor-cell invasion, Int. J. Cancer 65 (1996) 627–632.
[20] M. Mukai, H. Nakamura, M. Tatsuta, T. Iwasaki, A. Togawa, F. Imamura, H. Akedo, Hepatoma cell migration through a mesothelial cell monolayer is inhibited by cyclic AMP-elevating agents via a Rho-dependent pathway, FEBS Lett. 484 (2000) 69–73.
[21] H. Suwa, G. Ohshio, T. Imamura, G. Watanabe, S. Arii, M. Imamura, S. Narumiya, H. Hiai, M. Fukumoto, Overexpression of the RhoC gene correlates with progression of ductal adenocarcinoma of the pancreas, Br. J. Cancer 77 (1998) 147–152.
[22] K.L. van Golen, Z.F. Wu, X.T. Qiao, L.W. Bao, S.D. Merajver, RhoC GTPase, a novel transforming oncogene for human mammary epithelial cells that partially recapitulates the inflammatory breast cancer phenotype, Cancer Res. 60 (2000) 5832–5838.
[23] Y. Shikada, I. Yoshino, T. Okamoto, S. Fukuyama, T. Kameyama, Y. Maehara, Higher expression of RhoC is related to invasiveness in non-small cell lung carcinoma, Clin. Cancer Res. 9 (2003) 5282–5286.
[24] A. Horiuchi, T. Imai, C. Wang, S. Ohira, Y. Feng, T. Nikaido, I. Konishi, Up-regulation of small GTPases, RhoA and RhoC, is associated with tumor progression in ovarian carcinoma, Lab. Invest. 83 (2003) 861–870.
[25] E.A. Clark, T.R. Golub, E.S. Lander, R.O. Hynes, Genomic analysis of metastasis reveals an essential role for RhoC, Nature 406 (2000) 532–535.
[26] A. Hakem, O. Sanchez-Sweatman, A. You-Ten, G. Duncan, A. Wakeham, R. Khokha, T.W. Mak, RhoC is dispensable for embryogenesis and tumor initiation but essential for metastasis, Genes Dev. 19 (2005) 1974–1979.
[27] N.A. Bhowmick, M. Ghiassi, A. Bakin, M. Aakre, C.A. Lundquist, M.E. Engel, C.L. Arteaga, H.L. Moses, Transforming growth factor-beta1 mediates epithelial to mesenchymal transdifferentiation through a RhoA-dependent mechanism, Mol. Biol. Cell 12 (2001) 27–36.
[28] S. Edlund, M. Landstrom, C.H. Heldin, P. Aspenstrom, Transforming growth factor-β-induced mobilization of actin cytoskeleton requires signaling by small GTPases Cdc42 and RhoA, Mol. Biol. Cell 13 (2002) 902–914.
[29] C.G. Kleer, K.L. van Golen, Y. Zhang, Z.F. Wu, M.A. Rubin, S.D. Merajver, Characterization of RhoC expression in benign and malignant breast disease: a potential new marker for small breast carcinomas with metastatic ability, Am. J. Pathol. 160 (2002) 579–584.
[30] H. Akedo, K. Shinkai, M. Mukai, Y. Mori, R. Tateishi, K. Tanaka, R. Yamamoto, T. Morishita, Interaction of rat ascites hepatoma cells with cultured mesothelial cell layers: a model for tumor invasion, Cancer Res. 46 (1986) 2416–2422.
[31] A. Masszi, C. Di Ciano, G. Sirokmany, W.T. Arthur, O.D. Rotstein, J. Wang, C.A. McCulloch, L. Rosivall, I. Mucsi, A. Kapus, Central role for Rho in TGF-β1-induced α-smooth muscle actin expression during epithelial-mesenchymal transition, Am. J. Physiol. Renal Physiol. 284 (2003) F911–F924.
[32] L. Vardouli, A. Moustakas, C. Stournaras, LIM-kinase 2 and cofilin phosphorylation mediate actin cytoskeleton reorganization induced by transforming growth factor-β, J. Biol. Chem. 280 (2005) 11448–11457.
[33] E. Sahai, C.J. Marshall, ROCK and Dia have opposing effects on adherens junctions downstream of Rho, Nat. Cell Biol. 4 (2002) 408–415.
[34] K.J. Simpson, A.S. Dugan, A.M. Mercurio, Functional analysis of the contribution of RhoA and RhoC GTPases to invasive breast carcinoma, Cancer Res. 64 (2004) 8694–8701.
[35] L. Wang, L. Yang, Y. Luo, Y. Zheng, A novel strategy for specifically down-regulating individual Rho GTPase activity in tumor cells, J. Biol. Chem. 278 (2003) 44617–44625.
[36] J.Y. Pille, C. Denoyelle, J. Varet, J.R. Bertrand, J. Soria, P. Opolon, H. Lu, L.L. Pritchard, J.P. Vannier, C. Malvy, C. Soria, H. Li, Anti-RhoA and anti-RhoC siRNAs Inhibit the proliferation and invasive-

ness of MDA-MB-231 breast cancer cells in vitro and in vivo, Mol. Ther. 11 (2005) 267–274.
[37] R.S. Muraoka, N. Dumont, C.A. Ritter, T.C. Dugger, D.M. Brantley, J. Chen, E. Easterly, L.R. Roebuck, S. Ryan, P.J. Gotwals, V. Koteliansky, C.L. Arteaga, Blockade of TGF-β inhibits mammary tumor cell viability, migration, and metastases, J. Clin. Invest. 109 (2002) 1551–1559.
[38] R.B. Lobell, C.A. Omer, M.T. Abrams, H.G. Bhimnathwala, M.J. Brucker, C.A. Buser, J.P. Davide, S.J. deSolms, C.J. Dinsmore, M.S. Ellis-Hutchings, A.M. Kral, D. Liu, W.C. Lumma, S.V. Machotka, E. Rands, T.M. Williams, S.L. Graham, G.D. Hartman, A.I. Oliff, D.C. Heimbrook, N.E. Kohl, Evaluation of farnesyl:protein transferase and geranylgeranyl:protein transferase inhibitor combinations in preclinical models, Cancer Res. 61 (2001) 8758–8768.
[39] T. Kusama, M. Mukai, T. Iwasaki, M. Tatsuta, Y. Matsumoto, H. Akedo, M. Inoue, H. Nakamura, 3-hydroxy-3-methylglutaryl-coenzyme a reductase inhibitors reduce human pancreatic cancer cell invasion and metastasis, Gastroenterology 122 (2002) 308–317.