Original Article

Characterization of the Roles of RHOC and RHOA GTPases in Invasion, Motility, and Matrix Adhesion in Inflammatory and Aggressive Breast Cancers*

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BACKGROUND: The 2 closely related small GTPases, RHOC and RHOA, are involved in mammary gland carcinogenesis; however, their specific roles in determining cancer cell adhesion and invasion have not been elucidated. **METHODS:** RHOA and RHOC are highly homologous, thereby posing a major challenge to study their individual functions in cancer cells. By selectively knocking down these proteins, we have been able to alternatively inhibit RHOC and RHOA, while preserving expression of the other rho protein. Quantitative analyses of the growth patterns and invasion in the aggressive estrogen receptor negative cell lines MDA-231 and SUM149 were carried out on collagen I and Matrigel substrates. **RESULTS:** RHOC, and not RHOA, modulates surface expression and colocalization of α2 and β1 integrins in MDA-MB-231 on collagen I. Neither RHOC or RHOA affected integrin expression in the inflammatory breast cancer cell line SUM149, further highlighting the different regulation of adhesion and motility in inflammatory breast cancer. **CONCLUSIONS:** This work shows that RHOC and RHOA play different roles in cell-matrix adhesion, motility, and invasion of MDA-MB-231 and reaffirms the crucial role of RHOC-GTPase in inflammatory breast cancer cell invasion. *Cancer* **2010;116(11 suppl):2768–82.** © *2010 American Cancer Society.*

KEYWORDS: RHOA, RHOC, motility, invasion, adhesion.

RHOC and RHOA belong to the Ras superfamily of small GTPases. The protein sequences of RHOA and RHOC are approximately 90% homologous, with the major divergence occurring at the carboxyl termini. In the human genome, the *RHOC* gene (*ARHC*) contains fewer introns. This has led to the hypothesis that *RHOC* may have originated as an incomplete duplication of the *RHOA* gene.[1] Because the RHO proteins (A, B, C) are closely related to RAS oncogenes, their roles in carcinogenesis have been studied extensively. Persistent activation of RHO transforms fibroblasts and RHO inhibition abrogates oncogenic RAS-mediated transformation in these cells.[2] In addition, it has been shown that RHO activation increases cell proliferation by downregulating the cyclin-dependent kinase inhibitors, $p21^{WAF1}$ and $p27^{KIP1}$.[2] Unlike the case of *RAS*, there are no known pathogenic mutations in the *RHO* genes.[1] Biologically relevant aberrant levels of RHO expression, however, are commonly found in many types of cancers. For example, RHOA overexpression has been described in breast cancer, testicular germ-cell tumors, and colon cancer.[3] Conversely, upregulation of RHOC is prevalent in inflammatory breast cancer, pancreatic cancer, hepatocarcinoma, and melanoma, with a strong association between expression levels and poor prognosis.[4-10] Indeed, ectopic expression of RHOC in nontransformed immortalized breast cells leads to a highly malignant phenotype.[11]

RHO proteins play prominent roles in regulating cytoskeletal organization, directional migration, and tumor cell motility.[12] RHO activation leads to F-actin stress fiber formation and the assembly of focal adhesion complexes.[12] Within the focal adhesion complexes, integrins interact specifically with extracellular matrix (ECM) components.[12-15] Integrin

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engagement reciprocally activates the RHO proteins and their downstream effectors.[16] The functions of integrins in tumorigenesis are extremely diverse because of the variety of combinations of integrin subunits and their overlapping spectrum of interactions with different types of ECM.[17] Although cancer cells are relatively anchorage-independent and can survive and proliferate without certain ECM components, they are able to also modulate expression of different integrin subunits to support or enhance proliferation, survival, and migration[17,18-20].

The α2β1 integrin, a collagen receptor in many cell types, plays an important role in cell differentiation and morphogenesis.[21] The expression levels of the α2 subunit are altered in different stages of cell differentiation.[21] Expression of integrin α2 is stringently regulated in the normal mammary gland: it is downregulated during lactation and is elevated in the nonpregnant gland.[22] In poorly differentiated breast cancer cells, endogenously induced expression of the α2 subunit reduced their metastatic potential.[23] Ablation of α2 expression by antisense α2 mRNA treatment altered breast cell adhesion and motility on collagen.[24] Overexpression of the α2 subunit in rhabdomyosarcoma cells also rendered the cells invasive.[25] Moreover, expression of the α2 subunit was aberrantly elevated in in vitro RAS-transformed mammary epithelial cells and osteogenic sarcoma cells.[26] Tumor promoters 12-*O*-tetradecanoylphorbol 13-acetate (TPA) and okadaic acid (OA) further enhanced α2 transcription in human osteosarcoma cells and melanoma cells.[27,28]

The β1 subunit of the α2β1 integrin is by far the best-studied integrin and a crucial subunit of many ECM receptors. β1 receptor signaling is required for cell proliferation, survival, and differentiation in normal mammary gland development in vivo.[29,30] Notably, β1 null mammary epithelial cells proliferate more slowly than controls and express significantly higher levels of $p21^{Cip1}$, a cyclin-dependent kinase (CDK) inhibitor.[30] In a transgenic mouse model of breast tumorigenesis, β1 was found to be essential for oncogenic transformation and the maintenance of proliferative capacity in vivo.[31] β1 signaling promoted the invasion and metastasis of squamous cell carcinoma and RAS/MYC-transformed fibroblasts.[32,33] In addition, ablation of β1 in keratinocytes severely affected the orientation of the actin cytoskeleton and polarized migration required for cell motility.[34]

In this study, we report that RHOC modulates the expression and localization of the integrin α2 and β1 subunits in the breast cancer cell line MDA-MB-231. We aimed to understand the molecular basis of the role of RHOC and RHOA in motility and invasion of breast cancer cells. After producing the crucial but challenging cellular reagents that harbored siRNAs specific for either RHOC or RHOA, we were able to show that RHOA knockdown cells were more invasive and proliferative than RHOC-RNAi–expressing cells. Moreover, the RHOC knockdown cells failed to adhere to collagen I and expressed low surface levels of α2 and β1 integrin subunits, providing an explanation for their reduced motility and invasiveness. Therefore, we conclude that RHOC and RHOA are playing different roles in regulating MDA-MB-231 motility and invasion through cell-matrix adhesion.

MATERIALS AND METHODS

RNAi Design and Plasmid Construction

To stably express RNAi targeted to RHOA or RHOC, the small interference (si)RNA expression vector, pSilencer hygro (Ambion, Austin, Tex) was chosen in this study. RNAi target sites (21 mer) on RHOA and RHOC genomic sequences were selected using Ambion's siRNA target selection Web tool (http://www.ambion.com/techlib/misc/siRNA_design.html). The candidate sequences were validated by BLAST analysis (www.ncbi.nlm.nih.gov/BLAST) to eliminate the possibility of knockdown of genes other than RHOA and RHOC. The selected target sequences were incorporated separately into 55-60 nt hairpin siRNA oligonucleotide pairs, which encode 19 mer hairpin sequences specific to the mRNA target, a loop sequence separating the 2 complementary domains, and a polythymidine tract to terminate transcription (www.ambion.com/techlib/misc/psilencer_converter.html).

The primer sets (*RHOC* 121: 5′-GATCCCGCTATATTGCGGACATTGAGTTCAAGAGACTCAATGTCCGCAATATAGTTTTTTGGAAA-3′

RHOC 280: 5′-GATCCCGCATTCCTGAGAAGTGGACCTTCAAGAGAGGTCCACTTCTCAGGAATGTTTTTTGGAAA-3′

RHOA 150: 5′-GATCCAGCAGGTAGAGTTGGCTTTTTCAAGAGAAAAGCCAACTCTACCTGCTTTTTTTGGAAA-3′

RHOA 428: 5′-GATCCGGCAGAGATATGGCAAACATTCAAGAGATGTTTGCCATATCTCTGCCTTTTTTGGAAA-3′) were annealed onto *HindIII-BamHI* digested pSilencer Hygro according to the

manufacturer's instructions. The negative control scrambled siRNA (Ambion) on pSilencer was included in this study as a negative control.

Cell Culture

SUM149 cells (http://www.asterand.com/Services/RepositoryServices/149PT.asp) were cultured in Ham F12 (Mediatech, Manassas, Va) supplemented with 5% FBS (Invitrogen, Carlsbad, Calif), insulin and hydrocortisone (Sigma, St. Louis, Mo) at 37°C under 10% CO_2. MDA-MB-231 (American Type Culture Collection, Manassas, Va) was cultured in MEM (Mediatech) supplemented with Earle salt, L-glutamine and 10% fetal bovine serum (FBS; Invitrogen) at 37°C under 10% CO_2. The plasmids described above were transfected into SUM149 or MDA-MB-231 using GeneJammer transfection reagent (Stratagene, La Jolla, Calif). Stable transfectants were developed as previously described[35] and maintained in the medium supplemented with 150 μg/mL Hygromycin (Invitrogen).

Western Analysis

Cells were washed in cold phosphate-buffered saline (PBS) and lysed in lysis buffer for RHOC (10% glycerol, 50 mM Tris pH 7.4, 100 mM NaCl, 1% NP-40, 2 mM $MgCl_2$, 1 μg/mL leupeptin, 1 μg/mL aprotinin, 1 mM PMSF) or in RIPA buffer on ice for 5 minutes. After centrifugation for 5 minutes at top speed at 4°C, cleared lysates were collected, electrophoresed onto SDS-PAGE (60 μg of protein each lane), and transferred to polyvinylidene fluoride (PVDF) membrane using a semidry apparatus (BioRad, Hercules, Calif). Western analysis was performed as previously described[35] with the anti-RHOC rabbit polyclonal antibody (Santa Cruz Biotechnology, Santa Cruz, Calif) at 1:2000 dilution, the anti-RHOA mouse monoclonal antibody (Santa Cruz) at 1:1200 dilution, the anti-α2 polyclonal antibody (Santa Cruz) at 1:200 dilution, the anti-β1 polyclonal antibody (BD Bioscience, Franklin Lakes, NJ) at 1:500 dilution, the anti-α5 polyclonal antibody (Santa Cruz) at 1:200 dilution, or anti-β-actin goat antibody (Sigma) at 1:2000 dilution.

RHO Pulldown Assay

RHO pulldown assays were performed per manufacturer's instructions (Upstate Charlottesville, Va). Cells were cultured in 150 mm dishes to 85%-90% confluence. The dishes were rinsed twice with ice-cold Tris-buffered saline, and 0.5-1 mL of ice-cold Mg^{2+} lysis/wash buffer (25 mM HEPES, pH 7.5, 150 mM NaCl, 1% Igepal CA-630, 10 mM $MgCl_2$, 1 mM EDTA, 2% glycerol, 10 μg/mL aprotinin and 10 μg/mL leupeptin) was added to the plates on ice. Cells were scraped off the plates using a cell scraper. The lysates were transferred to eppendorf tubes and incubated for 15 minutes at 4°C with agitation. A fraction of the lysate (250 μL) was incubated with 30 μg of the GST-rhotekin agarose beads overnight at 4°C with agitation. The beads were spun down by brief centrifugation (14,000 rpm for 10 seconds) at 4°C. The supernatant was removed, and the beads were washed 3 times with 1X Mg^{2+} lysis/wash buffer (0.5 mL) and then resuspended in 2X Western buffer and boiled for 5 minutes. The supernatant was collected and loaded to an SDS-PAGE gel for Western analysis with RHOA and RHOC.

Immunofluorescence Staining of Cultured Cells

Cells were grown on collagen I-coated chamber slides for 24 hours and washed with PBS followed by fixation with 4% paraformaldehyde in PBS for 10 minutes. Fixed cells were washed in PBS-glycine (10 mM) for 5 minutes, incubated with 2% goat serum in PBS for 1 hr to block nonspecific binding, and permeabilized with 0.1% Triton X-100 in PBS-2% goat serum. For stress fiber staining, fixed cells were permeabilized with 0.1% Triton X-100 in PBS for 5 minutes, incubated with 1:100 dilution of Alexa-Fluor 568-conjugated phalloidin (Molecular Probes) for 15 minutes, and washed 3 times in PBS. To observe the focal adhesion points, a 1:200 dilution of the anti-FAK rabbit polyclonal antibody (Santa Cruz) was used as the primary antibody and 1% bovine serum albumin (BSA) was used as a blocking reagent in place of goat serum. For double staining of integrins, a 1:1000 dilution of the anti-α2 mouse monoclonal antibody (JBS2 clone; Chemicon, Billerica, Mass), a 1:1000 dilution of the anti-α5 mouse monoclonal antibody (NK1-Sam-1 clone; Chemicon), a 1:1000 dilution of the anti-β1 rat monoclonal antibody (AIIB2, a kind gift from Dr. Lilli Petruzzelli, University of Michigan), a 1:500 dilution of the anticofilin 2 rabbit polyclonal antibody (Upstate), or a 1:1000 dilution of the mouse IgG2a or IgG2b (Chemicon) in PBS-2% goat serum were added to each slide and allowed to stain for overnight at room temperature. The slides were subsequently washed 3 times in PBS-2% goat serum and stained with 1:1000 dilution of Alexa Fluor 488-conjugated antirat secondary antibody (Molecular Probes) and 1:1000 dilution of Alexa Fluor 568-conjugated antimouse secondary antibody (Molecular Probes), followed by 3 washes in PBS-2% goat serum. DAPI (4′,6-diamidino-2-

phenylindole; Molecular Probes) was used for nuclear counterstaining. All the slides were mounted in Prolonged Gold Antifade reagent (Molecular Probes) and observed under an Olympus FV-500 Confocal Microscope (Olympus, Melville, NY) at the Microscopy and Image Analysis Laboratory at the University of Michigan Medical School (CDB MIL).

Monolayer Growth Rate

Monolayer culture growth rate was determined by the degree of conversion of 3-(4,5-Dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) (Sigma) to a water-insoluble formazan by viable cells.[11] Five hundred cells in 200 mL of medium were plated in 96-well plates and grown under normal conditions. At various time points, cultured cells were treated with 40 mL of 5 mg/mL MTT for 1 hour at 37°C. The MTT-containing medium was aspirated and 100 μL of dimethyl sulfoxide (DMSO) (Sigma) were added to lyse the cells and solubilize the formazan. Absorbance values of the lysates were determined on a Dynatech MR 5000 microplate reader at 595 nm.

Random Motility Assay

Random cell motility was determined by using a motility assay kit (Cellomics, Pittsburg, Pa). Cells were harvested, washed with PBS, suspended in serum-free medium, and plated on top of microscopic fluorescent beads evenly distributed on a well of a 96-well plate coated with collagen I (BD Biocoat, Mississauga, Ontario, Canada). After a 24-hour incubation period, cells were fixed, and areas of clearing in the fluorescent bead field corresponding to phagokinetic cell tracks were quantified using NIH Image Version 1.63.

Boyden Chamber Invasion Assay

Invasion assays were performed on 24-well culture dishes in accordance with manufacturer's instructions (Chemicon). The inserts within each dish were equipped with a polycarbonate membrane on the bottom coated with a thin layer of extracellular matrix proteins (Matrigel, Mississauga, Ontario, Canada). In brief, the inserts were rehydrated in serum-free media. Cells were washed in PBS and resuspended in serum-free medium at a concentration of 5×10^5 cells/mL and 0.5 mL of cell suspension was added to the insert. Wells in the culture dish were filled with serum-containing medium. The dish was incubated for 24 hours at 37°C at 10% CO_2. The cell suspension was aspirated and noninvasive cells were removed from the insert using a cotton swab. The inserts were bathed in the staining solution for 20 minutes and then rinsed in water. Stained cells, which invaded through Matrigel, were counted under an Olympus inverted microscope.

Matrix Adhesion Assay

Adhesion assays were performed on 96-well culture strips precoated with collagen I per manufacturer's instructions (Chemicon). The wells were rehydrated in serum-free media. Cells were washed in PBS and resuspended in serum-free medium supplemented with 0.1% BSA at a concentration of 10^6 cells/mL, and 0.1 mL of cell suspension was added to each well. The strips were incubated for 1 hour at 37°C at 10% CO_2. The wells were aspirated, washed 3 times in PBS, and stained with the staining solution (0.2% crystal violet in 10% ethanol) for 5 minutes. Excess dye was removed by 3 washes of PBS and stained cells were scored by dissolving the dye in 10% acetic acid for OD measurement at 570 nm.

RESULTS

Development and Characterization of SUM149 and MDA-MB-231 Clones Stably Expressing RNAi Against RHOA or RHOC

To elucidate the roles of RHOA and RHOC in aggressive breast cancers, we designed hairpin siRNAs (sequences listed in the Materials and Methods section) to specifically and selectively target RHOA or RHOC. As cellular reagents, we chose to study the parental lines SUM149, derived from an inflammatory breast cancer (IBC) specimen and MDA-MB-231 derived from an estrogen receptor negative, metastatic breast cancer. The oligonucleotide pairs of hairpin siRNA RNAi-150 and RNAi-428 for RHOA, RNAi-121 and RNAi-280 for RHOC were ligated into pSilencer-Hygro vector (Ambion). These vectors were transfected into SUM149, an IBC cell line, and MDA-MB-231, known as a highly aggressive, estrogen receptor-negative, non-IBC breast cancer cell line. The hygromycin resistance marker in the vector allowed for the selection of stable clones expressing RNAi. Western analysis was used to identify hygromycin-resistant clones with very low RHOA or RHOC expression. All the RHOC knockdown clones of MDA-MB-231 were originally transfected with RNAi-121, while the SUM149 subclones were RNAi-280 transfectants only. For further characterization, we chose 2 stable RNAi clones against RHOA and 2 against RHOC in each cell line. We have

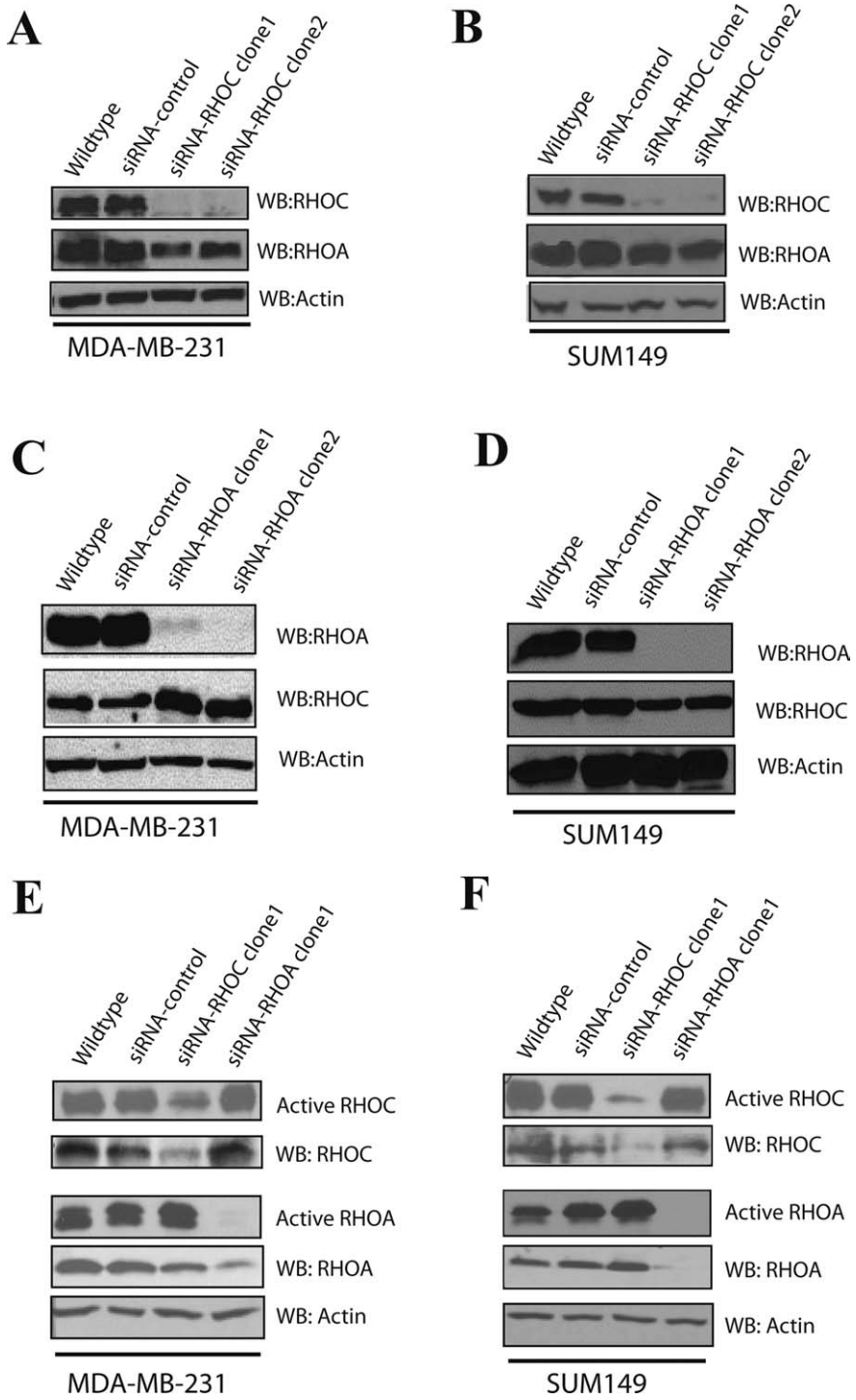

Figure 1. Expression of RHOA and RHOC in RHOC-siRNA–expressing clones of SUM149 and MDA-MB-231 is illustrated. (A,B) RHOC and RHOA protein expression of the RHOC-siRNA-expressing clones was confirmed by Western analysis using the anti-RHOA monoclonal antibody and anti-RHOC polyclonal antibody. Cleared lysates (50 μg of protein each) were electrophoresed onto SDS-PAGE and transferred to polyvinylidene fluoride membrane for Western analysis. The protein levels of β-actin were used as loading controls. (C,D) Protein expression of RHOA and RHOC in RHOA-siRNA expressing clones of SUM149 and MDA-MB-231. (E,F) RHOA and RHOC activity in the RHOA- or RHOC-knockdown cells. Cells were lysed and incubated with the GST-rhotekin RBD agarose beads overnight at 4°C with agitation. The beads were washed 3 times then resuspended in 2X Western buffer and boiled for 5 minutes. Approximately 1 of 3-1 of 4 of the supernatant (equal to 700-800 μg of total protein) was loaded onto an SDS-PAGE gel for Western analysis with RHOA and RHOC.

confirmed that the stably transfected siRNA against RHOA or RHOC effectively knocked down target protein expression (Fig. 1). Western analysis confirmed that RHOC protein levels among the RHOC siRNA clones of both breast cancer cell lines were 85% lower than in untransfected and siRNA negative controls (Fig. 1A, 1B). The siRNA knockdown of RHOC was effective and selective in that it did not significantly affect RHOA expression in most cases (Fig. 1A, B, E, F). The RHOA protein levels were either stable (Fig. 1B, E, F) or slightly lower than the controls in the RHOC knockdown clones of MDA-MB-231 (Fig. 1A). Conversely, we have also demonstrated that our hairpin RHOA siRNA dramatically reduced RHOA expression but did not significantly decrease RHOC expression (Fig. 1C, D, E, F). We observed that RHOC protein levels slightly increased in the siRNA-RHOA MDA-MB-231 clones (Fig. 1C, E).

The SUM149 and MDA-MB-231 Clones Stably Expressing RNAi Against RHOA or RHOC Specifically Knockdown RHOA or RHOC Activity

We expected that the knockdown clones with low expression levels of RHOA or RHOC would yield low activity of RHOA or RHOC. The Rho pulldown assay (affinity precipitation) was performed to demonstrate the activity levels of RHOA and RHOC. In brief, these clones (1 for each group) were expanded in 150 mm dishes, lysed, and incubated with the RHO-binding domain (RBD) of rhotekin, a paradigm of a RHOC effector, immobilized to agarose beads (Upstate). The active RHOA or RHOC protein in a GTP-bound form could interact specifically and tightly to rhotekin RBD during washes, and would be released by boiling in Western sample buffer. For control purpose, a fraction of the sample was subjected to Western analysis of RHOC and RHOA. The levels of active RHOC in the siRNA-RHOC–expressing MDA-MB-231 clones decreased >30% compared with untransfected and the siRNA negative controls (Fig. 1E), whereas the RHOC-knockdown clone of SUM149 showed a 70% decrease in active RHOC (Fig. 1F). As expected, the activity of RHOA remained constant in the siRNA-RHOC clones compared with the controls (Fig. 1E, F). The siRNA knockdown of RHOA in both lines effectively and specifically decreased RHOA activity in comparison to the control clones (Fig. 1E, F). Thus, we have confirmed that the RHOA- or RHOC-knockdown clones contained significantly lower levels of active RHOA or RHOC (Fig. 1E, F), respectively.

Stable Clones Expressing RNAi Against RHOC or RHOA Show Reduced Invasion, Motility, and Monolayer Growth Rate

Our initial hypothesis was that RHOA or RHOC knockdown in cancer cells would decrease their proliferation rate. Mammary epithelial cells stably transfected with RHOC and constitutively active RHOC showed a 2-fold increase in the monolayer growth assay as compared with the vector-only controls.[35] The results indicate that RHOA and RHOC knockdown clones exhibit different rates in MTT assays (Fig. 2A, B). SUM149 and MDA-MB-231 expressing RHOC-siRNA grew very slowly, at a rate that is 4-fold slower than untransfected and control siRNA samples (Fig. 2A, B). In contrast, the specific knockdown of RHOA did not affect cancer cell growth as prominently as the RHOC knockdown (Fig. 2A, B). These results suggest differential effects of RHOA and RHOC on signaling to the cell cycle or time of passage through the cycle.

Overexpression of RHOC in immortalized breast cells is known to increase cell invasion and motility, 2 important phenotypic parameters for quantifying the metastatic potential of cancer cells in vitro.[11,35] RHOA overexpression was also observed in several breast cancer cell lines.[3,36] In this study, we proposed that decrease of RHOC or RHOA below their regular levels in breast cancer cells would reduce cell invasiveness and motility. Indeed, specific knockdown of RHOA or RHOC rendered cancer cells less motile on collagen I in random motility assays, as showed in Figure 2C days. It is worth noting that we found that RHOC siRNA-expressing cells exhibited approximately 50% less motility than their RHOA siRNA-expressing counterparts (Fig. 2C, D). SUM149 and MDA231 cells expressing RHOC or RHOA siRNA also showed significant decreases of invasion in an invasion assay with Boyden chambers coated with Matrigel (Fig. 2E, F). Because Matrigel comprises similar components as the basement membrane, including laminin and collagen IV,[37] our results support that breast cancer cells with low RHOA or RHOC expression have reduced ability to penetrate the membrane.

RHOC-Silenced MDA-MB-231 Line Has Very Low Adhesion on Collagen I

During the course of this work, we extensively investigated the phenotypic differences between the RHOA- and RHOC-knockdown clones in SUM149 and MDA-MB-231. Because the knockdown clones exhibited low motility on collagen I, we examined their adhesion on collagen I using the cell-ECM adhesion assay (Chemicon).

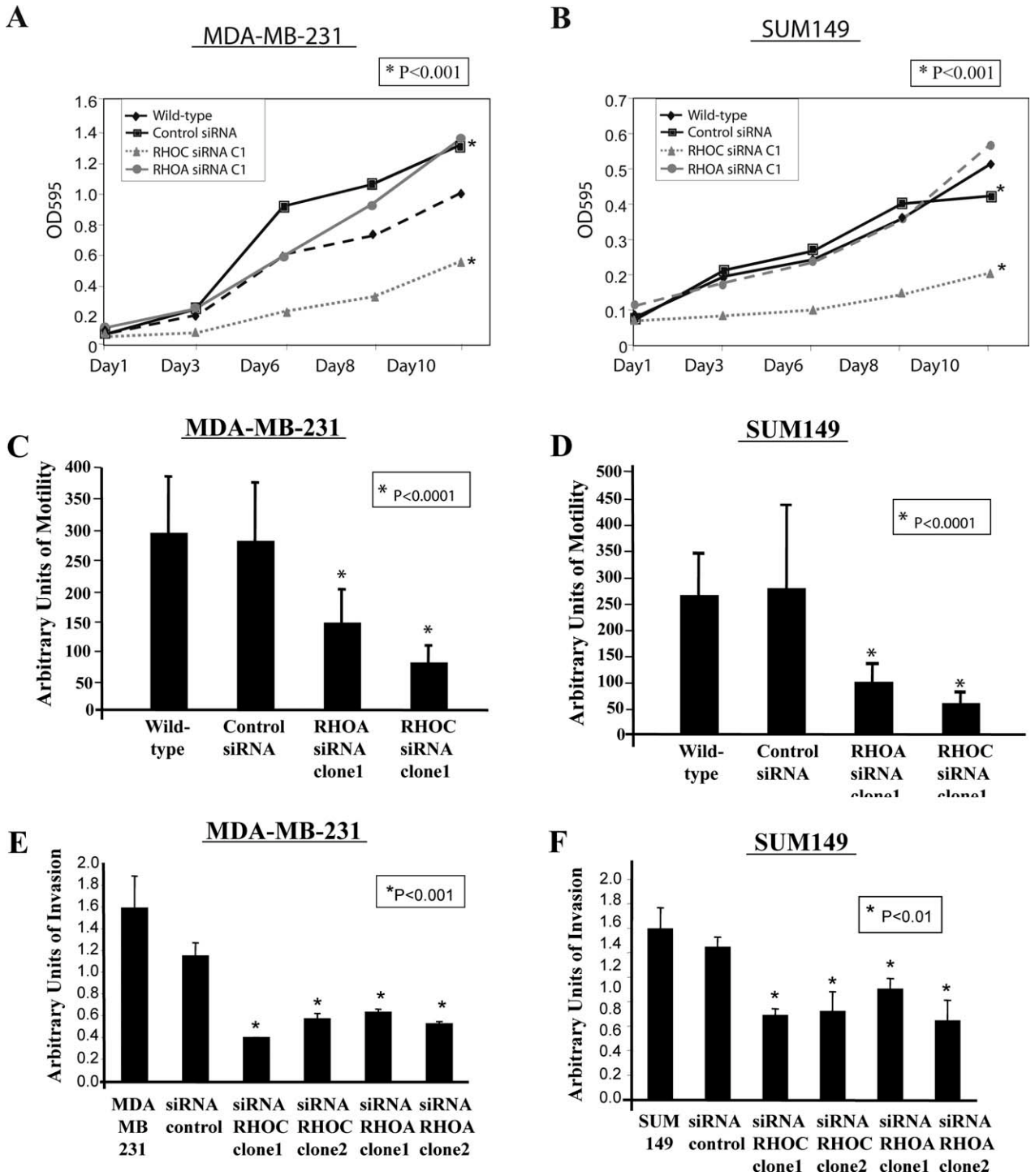

Figure 2. Characterization of malignant phenotypes in the small interference (si)RNA-expressing clones and the controls is illustrated. (A,B) Monolayer growth rate of RHOC-knockdown or RHOA-knockdown clones is depicted. RHOC transfectants (500 cells), taken at Day 1, 3, 6, 8, and 10, were incubated with 3-(4,5-Dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT), solubilized in dimethyl sulfoxide (DMSO), and measured absorbance at 595 nm. (C,D) The RHOC- or RHOA-specific knockdown clones showed decreased motility in random motility assay. Cells were trypsinized, washed with PBS, and suspended in serum-free medium, and plated on top of a field of microscopic fluorescent beads. After a 24-hour incubation period, cells were fixed, and areas of clearing in the fluorescent bead field corresponding to phagokinetic cell tracks were quantified using NIH Image. (E,F) The RHOC- or RHOA-specific knockdown clones of SUM149 and MDA-MB-231 had reduced invasion using Boyden chamber quantitative invasion assay. Cells were resuspended in serum-free medium and seeded in the chemotaxis chamber coated with basement membrane extracellular matrix. Cells penetrating through the artificial basement membranes were stained and scored under a phase contrast microscope, or were treated with 10% acetic acid to elute the dye for optical density (OD) readings at 560 nm. *p value versus untransfected control or the siRNA control.

Indeed, the RHOC-knockdown MDA-MB-231 cells adhered approximately 10-fold less than the controls on collagen I (*P* value <.01) (Fig. 3A). In contrast, knocking down of RHOA in MDA-MB-231 did not affect cell adhesion as significantly as RHOC did, because adhesion was lowered only to 20% of the control levels in the RHOA-RNAi cells (Fig. 3A). This suggests that RHOA and RHOC also play different roles in regulating cell adhesion, motility, and invasion on collagen I. Interestingly, no significant difference was observed between the SUM149 RHOA-RNAi or RHOC-RNAi clones in their adhesion on collagen I (Fig. 3B). This further suggests a difference between mechanism of cell adhesion, motility, and invasion in the 2 malignant breast cancer cell lines we tested. On the basis of these results, we proposed that the knockdown of RHOC in MDA-MB-231 compromised the function of the collagen I receptor and resulted in decreased collagen I adhesion and motility.

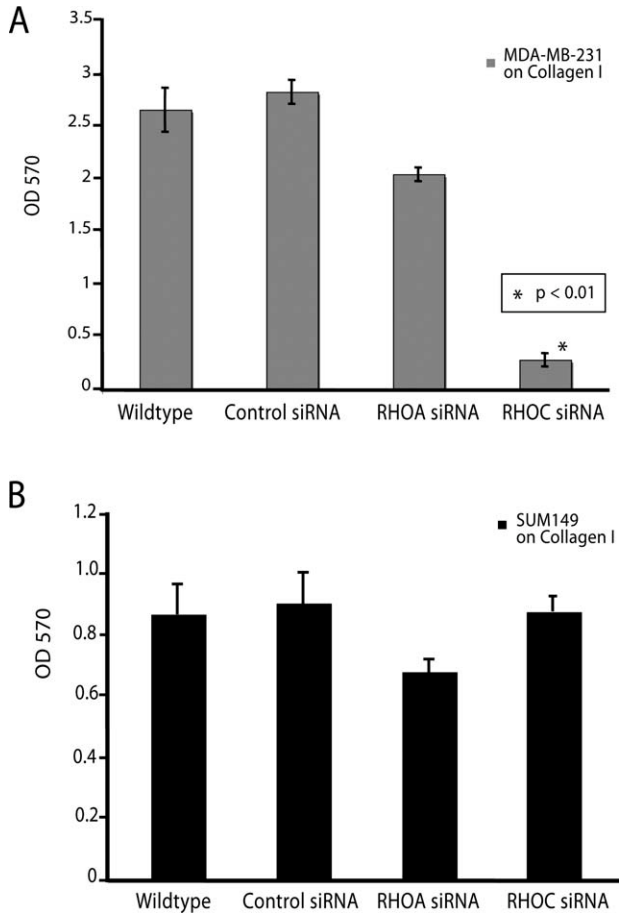

Figure 3. Collagen I adhesion of the small interference (si)RNA-expressing clones and the controls of MDA-MB-231 and SUM149 are depicted. (A, B) Adhesion assay on collagen I of the MDA-MB-231 (A) and SUM149 clones (B) was conducted using Cytomatrix strips (Chemicon). The adhered cells were stained and scored using OD measurement at 570 nm. **p*: the RHOC values versus the siRNA control.

Stable Clones Expressing RNAi Against RHOC or RHOA in MDA-MB-231 Show Reduced Stress Fiber Assembly and Drastically Fewer Focal Adhesions

RHOC overexpression in mammary epithelial cells induces the assembly of actin filaments and the formation of multiple focal adhesions.[11,35] Given this finding, we proposed that reduced RHO expression in highly motile cancer cells would significantly decrease stress fiber formation as well as the number and size of focal adhesions. The wildtype and control-RNAi transfected cells cultured on collagen I-coated glass slides displayed abundant stress fibers in star-like foci across the cells[38] (Fig. 4A, arrows). These cells had formed extensive focal adhesions around the borders to support their attachment to the matrix (Fig. 4B). Cells expressing RHOA-siRNA showed fewer and thinner actin filaments within the cytoplasm, but they lacked strong stress fibers and focal adhesions (Fig. 4A, B). Also, some residual stellae foci of the actin filaments were observed in these cells (Fig. 4A, arrows). In contrast, the RHOC-knockdown cells showed some cortical actin staining with no stress fiber formation and small focal adhesions (Fig. 4A, B). They failed to display the star-like foci of actin filaments, suggesting that these cells were less contractile (Fig. 4A). In summary, we have found that RHOC-knockdown blocked the assembly of stress fibers and contraction in MDA-MB-231 cells, while silencing of the RHOA gene did not completely disrupt F-actin assembly and contraction. These results further suggest intrinsic differences between RHOA and RHOC signaling in cytoskeleton assembly and cell contraction. This is consistent with our findings in the adhesion assay.

RHOC Knockdown Impairs α2β1 Expression and Surface Localization in MDA-MB-231 But Not in SUM149

The RHOC-knockdown MDA-MB-231 cells showed very low adhesion to collagen I. Because the α2β1 integrin is a major collagen receptor for luminal cells in mammary glands, we sought to examine the protein expression levels of the α2 and β1 subunits in the cell lysates. Expression of α2 and β1 was downregulated by 50% in RHOA- and RHOC-knockdown MDA-MB-231 clones (Fig. 5). The α5 subunit, part of the fibronectin receptor α5β1, however, was largely unaffected in all the MDA-MB-231

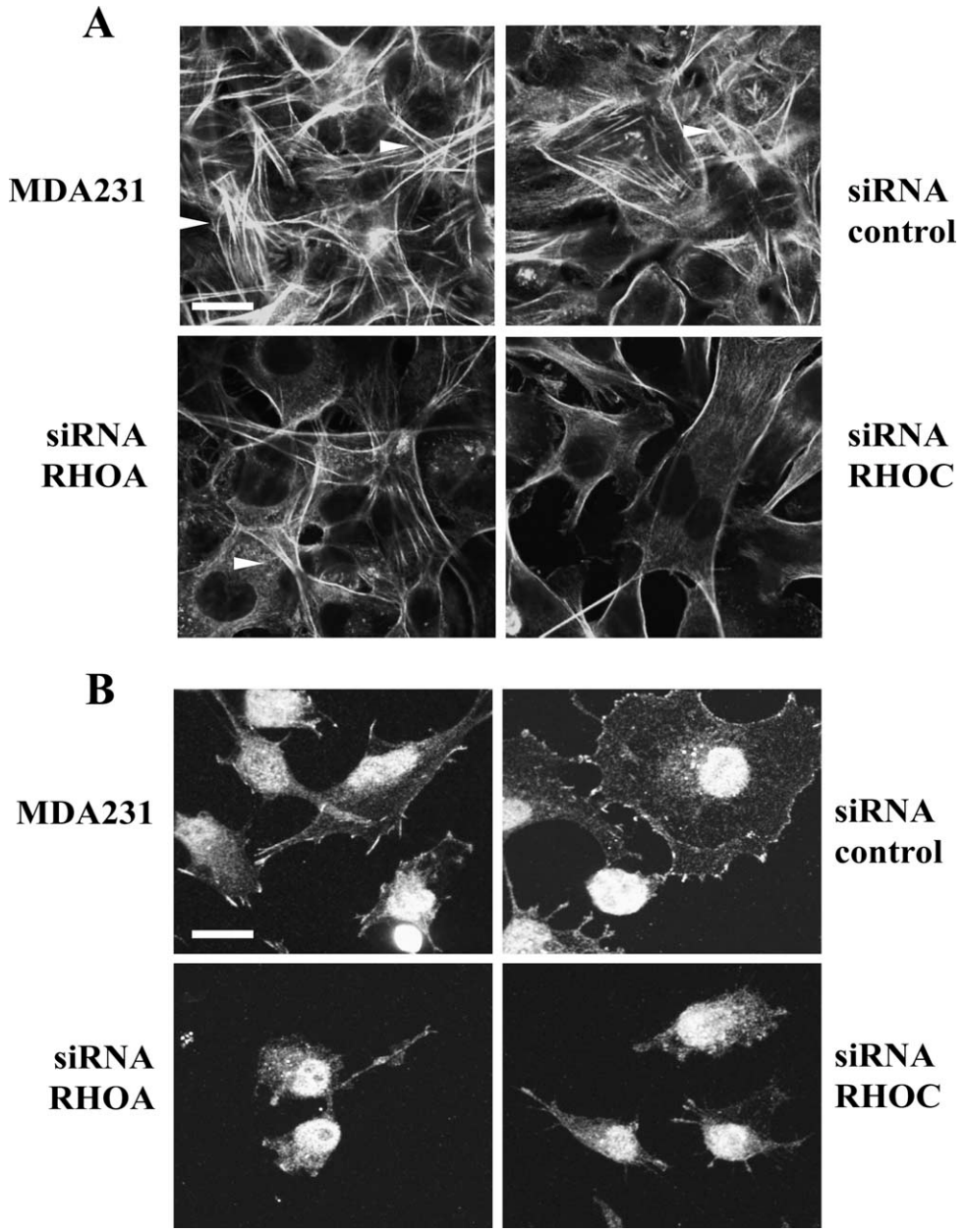

Figure 4. Stress fiber and focal adhesion formation of the small interference (si)RNA-expressing clones and the controls are depicted. (A,B) Confocal microscopy of the siRNA-transfected MDA-MB-231 cells stained with rhodamine-phalloidin (A) or FAK (B) to observe stress fibers and focal adhesion points. The star-like foci of stress fibers in the controls and the RHOA-knockdown cells were marked with white arrows. Bar, 50 μm.

sublines (Fig. 5). Conversely, the integrin expression patterns of α2 and β1 in all the SUM149 clones were nearly identical (Fig. 5). With the protein levels of β1 being somewhat increased in the RHOA- and RHOC-knockdown SUM149 clones (Fig. 5).

As the cell content of the individual integrin subunits may not reflect the actual amount of functional integrin receptors on the plasma membrane, we used immunofluorescence staining of the α2 and β1 subunits on the cell surface to detect the presence of presumed functional α2β1 receptors (Fig. 6A). In the untransfected MDA-MB-231 and control siRNA samples, we found that the integrin subunits strongly colocalized on the cell membrane in a ring-like pattern (Fig. 6A). The colocalization is consistent with the model that activated and engaged integrin receptors cluster together and interact

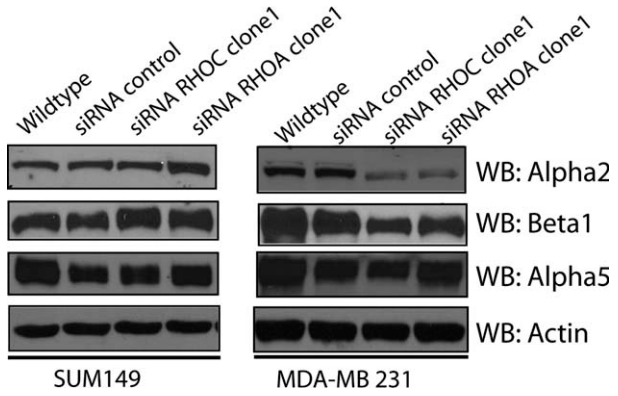

Figure 5. Integrin α2 and β1 expression in the whole cell lysates of MDA-MB-231 clones is depicted. Protein levels of integrins α2, α5, β1, and actin (loading control) were measured using 50 μg of lysates in RIPA buffer. Polyclonal antibodies against α2, α5, and β1 were used for the Western analysis.

with the ECM on the cell surface.[17] The RHOA-knockdown cells showed both weaker staining and membrane colocalization of α2 and β1, at significantly lower levels than the 2 control lines (Fig. 6A). In the RHOC-knockdown line, the α2 and β1 staining was even lower and the membrane localization was completely disrupted (Fig. 6A). We observed some α2 and β1 staining within the cytoplasm (Fig. 6A). Conversely, the surface localization of the α5 integrin showed no difference under any conditions (Fig. 6B). The RHOC-knockdown cells showed faint β1 staining, so consequently the colocalization of α5β1 was not prominent (Fig. 6B).

In comparison to the MDA-MB-231 and siRNA-RHOC cells and the SUM149 and siRNA-RHOA cells, the RHOC-knockdown SUM149 cells showed no significant change in cell adhesion on collagen I. We further examined the immunofluoresence patterns of integrins α2, α5, and β1 and found that their membrane localization was nearly identical among all the SUM149 clones (Fig. 7A, B). Thus, knockdown of RHOC in the SUM149 IBC cells did not affect the expression or colocalization of α2 and β1 in SUM149 (Fig. 7A). The control staining with cofilin-1 and the isotype-matching mouse IgGs was also performed in these lines, both showing very low background staining (data not shown).

In conclusion, we observed that the major difference between the RHOA- and RHOC-knockdown MDA-MB-231 lines was their ability to adhere on collage I. Consistent with this observation, we observed weak staining and membrane localization of integrin α2 and β1 in RHOC-knockdown cells, unlike the stronger staining and proper colocalization of the integrin subunits found in RHOA-knockdown cells. We conclude that RHOC, but not RHOA, plays a major role on MDA-MB-231 cell adhesion to collagen I. This work also suggested a difference between the roles of RHOA and RHOC in motility and invasion on collage I in the MDA-MB-231 line. In contrast, the protein expression levels and membrane localization of integrin α2 and β1 on collage I were not significantly affected in all the SUM149 lines, suggesting a different mechanism of invasion in SUM149.

DISCUSSION

RHO GTPase activation and signaling is highly complex, given the finding that RHO transduces signals to various immediate downstream effectors via direct binding. ROCK, CITRON, PKN, and RHOTEKIN are major effectors that regulate cell morphology, actin cytoskeleton assembly, gene expression, cell proliferation, and survival downstream of their interaction with RHO.[2] We sought to delineate the difference in RHOA- and RHOC-dependent signaling using RNA interference technology to specifically knockdown the expression of each GTPase in breast cancer cells. Compared with the well-studied RHOA, RHOC function and signaling had remained poorly understood. Evidence showed that RHOC appeared to have higher affinity and specificity to ROCK and CITRON than RHOA,[39,40] thereby hinting that the downstream RHOA phenotype may differ from RHOCs. The activation of ROCK by RHOC leads to disruption of adherens junctions.[40] ROCK activation by RHO/RHOC also contributed to increased cell motility, enhanced F-actin assembly, and carcinogenesis through activation of the LIM kinase-cofilin pathway.[16,40-43] Consistent with our results, Hakem and colleagues[44] recently established the RHOC-knockdown transgenic mice, which showed no significant defect in embryogenesis, development, and tumor initiation by polyoma virus middle T antigen. However, the *RHOC*$^{-/-}$ mice developed fewer instances of lung metastases of significantly smaller size. The authors hypothesized that RHOA is involved in tumor initiation and RHOC is crucial for tumor metastasis.[44]

In an attempt to identify molecules involved in RHOC downstream signaling, we had previously performed microarray analysis of RHOC-overexpressing MCF10A cells.[35] We found that overexpression of RHOC significantly increased the mRNA levels of *cyclin D1, fibronectin, VEGF-C, caveolin-2*, and *CXCL1*, and also decreased the levels of *IGFBP-2*.[35] These gene products, which are involved in cell cycle (*cyclin D1*), cell-

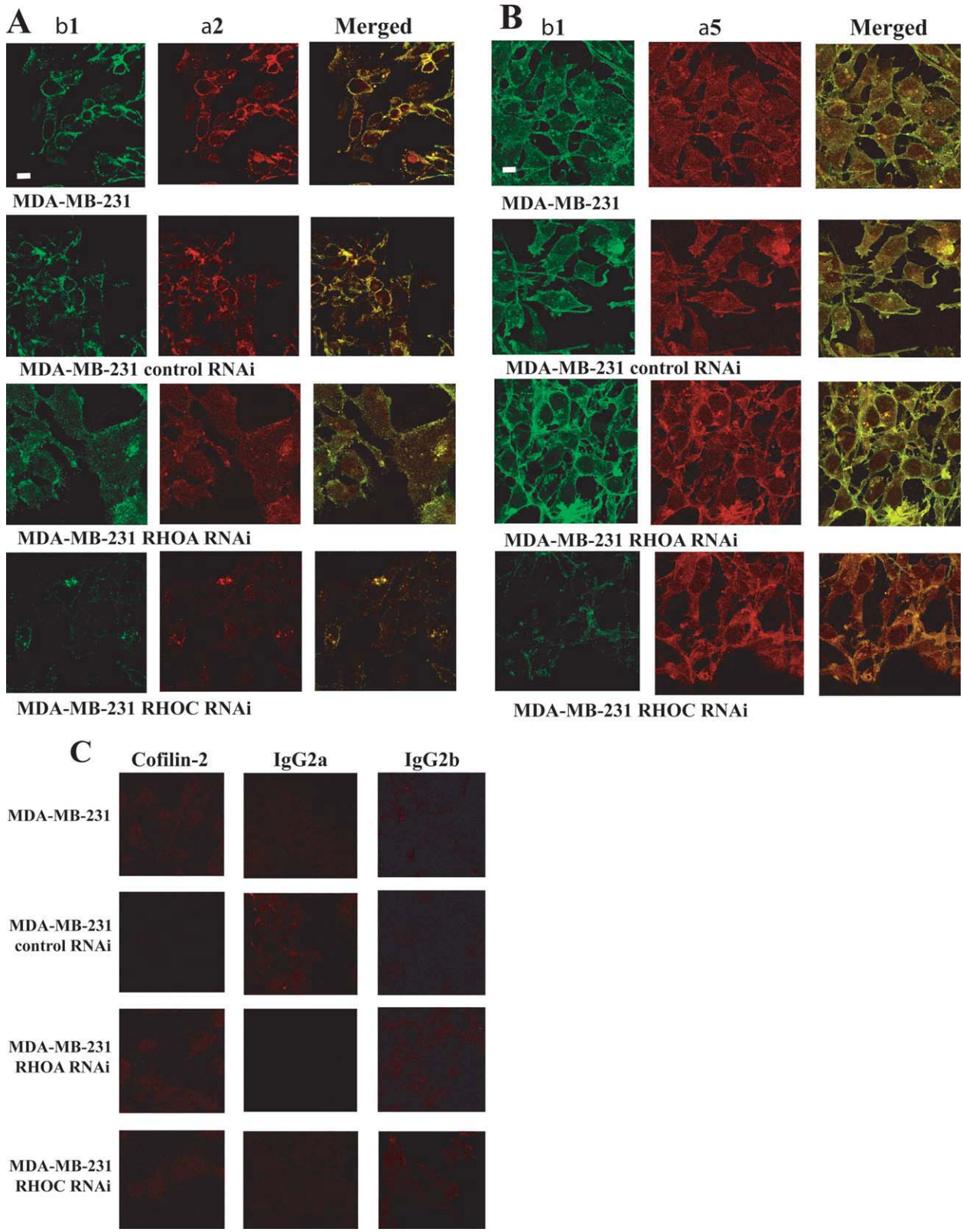

Figure 6. Immunofluorescence analysis of α2 and β1 integrins in the small interference (si)RNA-expressing MDA-MB-231 clones and controls is depicted. (A,B) Confocal microscopy of the RHOC- or RHOA-siRNA expressing MDA-MB-231 clones costained with anti-α2 and anti-β1 monoclonal antibodies (A), or with anti-α5 and anti-β1 monoclonal antibodies (B). Bar, 10 μm.

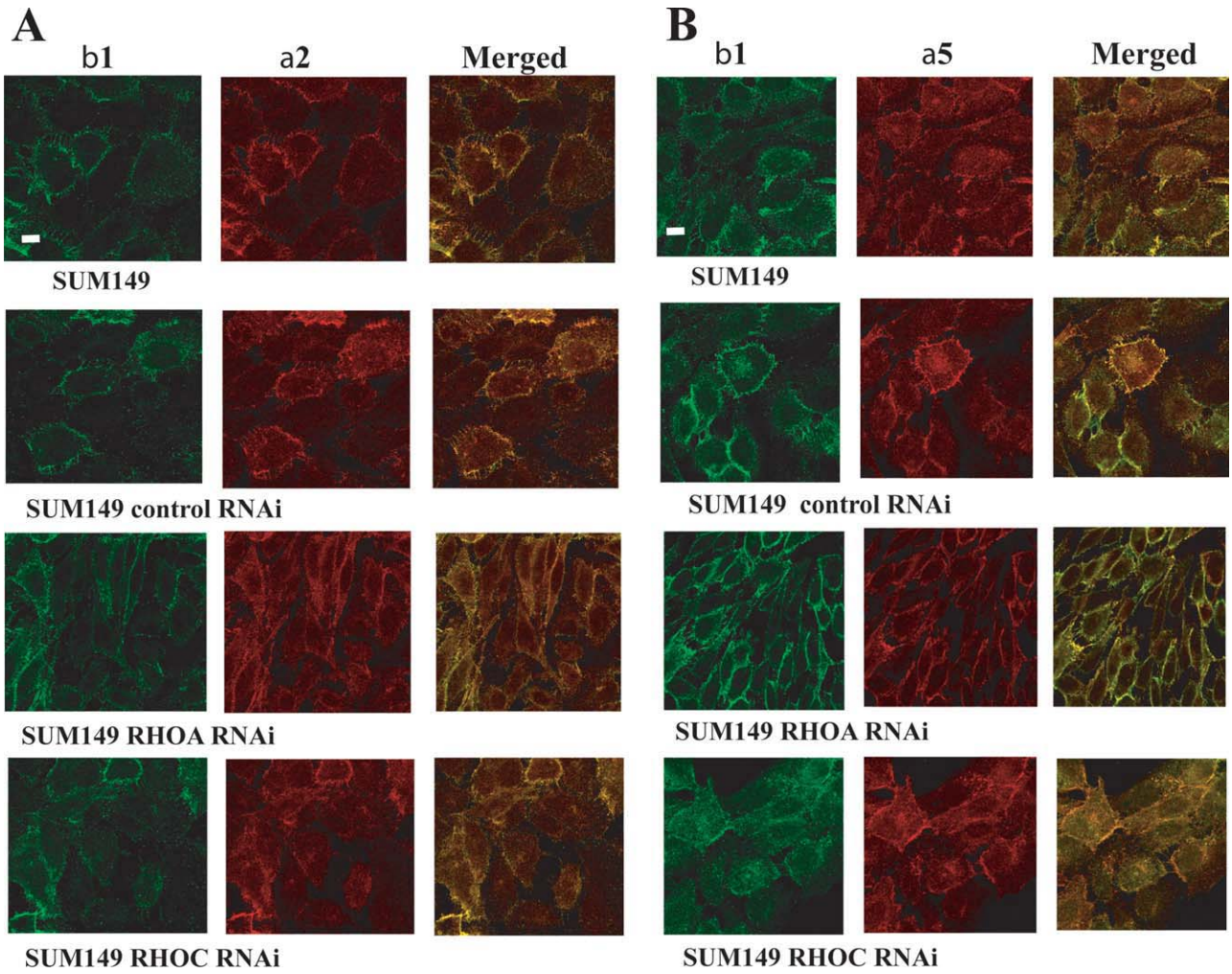

Figure 7. Immunofluorescence analysis of α2 and β1 integrins in the small interference (si)RNA-expressing SUM149 clones and controls is depicted. (A,B) Confocal microscopy of the RHOC- or RHOA-siRNA expressing SUM149 clones costained with anti-α2 and anti-β1 antibodies (A), or with anti-α5 and anti-β1 antibodies (B). Bar, 10 μm.

matrix adhesion (*fibronectin, caveolin*-2), and angiogenesis (*CXCL1* and *VEGF-C*), can contribute to the invasive, angiogenic, and motile phenotype of RHOC-overexpressing breast cells. Of particular interest is the role of RHOC in the regulation of cell-matrix adhesion, because one of the key functions of the RHO proteins is to respond to matrix adhesion signals relayed by integrins by promoting the assembly of focal adhesions and stress fibers.[12]

In this study, we sought to advance our understanding of signaling by RHOA and RHOC in MDA-MB-231 and SUM149, both estrogen receptor negative and poorly differentiated breast cancer cell lines, with very different clinical phenotypes: SUM149 is an inflammatory breast cancer cell line, whereas MDA231 is an aggressive non-IBC cell line. Knockdown of RHOA and RHOC in these 2 lines greatly reduced their metastatic phenotypic features, including proliferation, motility on collagen, and invasion into Matrigel. These results correlate well with previous findings for MDA-MB-231 cells.[45] In SUM159, a cell line from an anaplastic breast carcinoma, Simpson et al concluded that RHOA knockdown inhibited its metastatic phenotypes instead of RHOC.[46] Unless SUM159 is not representative of our findings because of its own particular genetic alterations, his result is not in agreement with our data and that of Hakem et al. In our hands, RHOC-specific knockdown appeared to have a quantitatively stronger effect in reducing motility ($P < .0001$), proliferation ($P < .001$), and invasion ($P < .001$), recapitulating the in vivo results.[44]

Our data also show that RHOC knockdown decreases surface expression and colocalization of the

integrin α2 and β1 subunits in MDA-MB-231, a poorly differentiated breast cancer line. Conversely, Zutter at al reported that forced expression of α2 in an α2 null, poorly differentiated breast cancer line Mm5MT reduced its metastatic potential.[23] At the first glance, our result appears to be inconsistent with the finding of Zutter et al. However, because of the pleiotropic effects of RHOC signaling, decreased expression of α2 and β1 by RHOC silencing is unlikely to be the sole mechanism leading to the reduction of MDA-MB-231 invasiveness. Indeed, we recently found that the RHOC-knockdown MDA-MB-231 cells displayed organized structures containing ZO-1, a tight junction marker, and bore a striking resemblance to the nontumorigenic MCF10A breast cells (Wu M, unpublished observations). This result strongly suggests that RHOC regulates not only cell-matrix adhesion but also cell-cell adhesion in the metastatic cascade.

Cell-matrix adhesion is crucial in several functions that comprise the metastatic process, such as local invasion, angiogenesis and colonization of distant sites by tumor metastasis.[47] Downregulation of integrin subunits contributes to a reduction of cell adhesion to the integrin-specific extracellular matrix. RHOC-knockdown MDA-MB-231 cells adhered very poorly on collagen I, a major ECM in interstitial matrix and bones. In addition, these cells did not move on collagen I-coated wells. RHOC-silenced cells had very weak α2β1 colocalization and focal adhesion, correlating with their low adhesion and motility on collagen. Indeed, other groups have shown that in prostate cancer, elevated levels of RHOC enhance collagen I-α2β1 signaling and promote tumor metastasis to bone matrix, which contains abundant collagen I.[48,49]

In integrin-matrix signaling, the surface amount, affinity, and avidity of the given integrin subunits is regulated by growth factor receptor pathways, RAS-MAPK pathway, and RHO pathway.[50] Recently, the oncogenic RAS-MAPK pathway was identified as an activator of RHOA, capable of activating the β1 integrin through FRA-1, a target of the MAPK cascade.[51] Crosstalk and/or feedback between the RAS and RHO pathways also enhance cell motility and proliferation.[52,53] In the breast cancer cell line T74D, α2β1- instead of α5β1 mediated cell motility was selectively enhanced by R-RAS and phosphatidylinositol 3-kinase (PI3-K).[54] In our work, we found that RHOC knockdown decreased total protein levels of the integrin α2 subunit and abolished its surface localization. Expression of α2 is regulated by the 2 oncogenic Ras isoforms, H-RAS and K-RAS,[26,28,55] suggesting that RAS-RHOC crosstalk/feedback may play a role in regulating the expression levels of the α2 integrin. This hypothesis is further supported by our previous result that RHOC overexpression in mammary epithelial cells triggered activation of the MAPK pathway responsible for motility, invasion and production of angiogenic factors.[56] In conclusion, our data defines the role of RHOC as a unique and dominant force in the modulation of adhesion and phenotypic metastatic features in breast cancer. We propose the RAS-RHOC crosstalk/feedback is the possible mechanism in the regulation of cell-matrix adhesion and integrin signaling of MDA-MB-231.

CONFLICT OF INTEREST DISCLOSURES

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