Knockdown of Contactin-1 Expression Suppresses Invasion and Metastasis of Lung Adenocarcinoma

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Abstract
Numerous genetic changes are associated with cancer cell metastasis and invasion. In search for key regulators of invasion and metastasis, a panel of lung cancer cell lines with different invasive ability was screened. The gene for contactin-1 was found to play an essential role in tumor invasion and metastasis. Suppression of contactin-1 expression abolished the ability of lung adenocarcinoma cells to invade Matrigel in vitro as well as the polymerization of filamentous-actin and the formation of focal adhesion structures. Furthermore, knockdown of contactin-1 resulted in extensive inhibition of tumor metastasis and in increased survival in an animal model. RhoA but not Cdc42 or Rac1 was found to serve a critical role in contactin-1-mediated invasion and metastasis. Contactin-1-specific RNA interference resulted in loss of metastatic and invasive capacity in both in vitro and in vivo models. This loss was overturned by constitutive expression of the active form of RhoA. Contactin-1 was differentially expressed in tumor tissues, and its expression correlated with tumor stage, lymph node metastasis, and patient survival. Contactin-1 is proposed to function importantly in the invasion and metastasis of lung adenocarcinoma cells via RhoA-mediated mechanisms. (Cancer Res 2006; 66(5): 2553-61)

Introduction
Lung cancer remains the leading cause of cancer death in industrialized countries. Metastasis, the major factor governing cancer patient mortality, is a complicated process that is currently uncontrolled (1). It is now widely accepted that malignant tumors contain heterogeneous populations of cells of varying metastatic potential (2). Consequently, highly and weakly metastatic cells in a given tumor should differ from each other with respect to biological properties, such as invasiveness, adhesiveness, motility, and proliferation capacity.

Understanding of the genetic alterations responsible for the molecular biological changes associated with human lung cancer pathogenesis has advanced during past decades. Considerable evidence exists to support the concept that each discrete step of metastasis is regulated by transient or permanent changes at the DNA, mRNA, and/or protein levels for different genes (3). Some of these molecules facilitate invasion and metastasis [e.g., receptors for laminin (4) and vitronectin (5), matrix metalloproteinases/MMPs (6, 7), and CD44 (8, 9)], whereas others inhibit these processes [e.g., cadherin (10), tissue inhibitors of MMPs (11, 12), nm23 (13), connective tissue growth factor/CTGF (14), and collapsing response mediator proteins/CRMP-1 (15)]. Understanding of the regulation of gene expression in poorly metastatic compared with highly metastatic cancer cells should facilitate the identification of genes associated with metastasis as well as the development of novel therapeutic and diagnostic applications, thereby improving clinical care of cancer patients.

Analysis of gene expression patterns has recently become possible through cDNA microarray techniques (16, 17). Microarray technology also represents a powerful tool for identifying oncogenes or tumor suppressor genes potentially involved in the etiology of lung cancer. In the present report, the cDNA microarray method, rather than the traditional paired comparison approach, was used to identify invasion-associated genes in a series of lung adenocarcinoma cell lines. Several genes critical to lung metastasis (14, 15) were identified using this approach. Here, a differentially expressed gene, contactin-1 (CNTN-1), was associated with invasive ability and reversely correlated with the prognosis of lung adenocarcinoma patients. The activity of the small GTP-binding protein, RhoA, was found to be required for CNTN-1-mediated filamentous-actin (F-actin) polymerization and cell invasion.

Materials and Methods
Cell culture, antibodies, and reagents. Lung adenocarcinoma cells were cultured as described previously (18). MAB904 mouse monoclonal anti-CNTN-1 and AF904 goat polyclonal anti-CNTN-1 antibodies were purchased from R&D Systems (Minneapolis, MN). Rhodamine-conjugated anti-phalloidin antibody was purchased from Molecular Probes (Eugene, OR). Goat polyclonal anti-CNTN-1, rabbit polyclonal anti-paxillin, anti-Rac1, mouse monoclonal anti-vinculin, anti-RhoA, and anti-Cdc42 antibodies were purchased from Santa Cruz Biotechnology (Santa Cruz, CA). Hoechst 33258 was obtained from Sigma (St. Louis, MO).

In vitro invasion assay and cell proliferation measurement. Modified Boyden chambers with filter inserts (pore size of 8 μm) coated with Matrigel (40 μg; Collaborative Biomedical, Becton Dickinson Labware, Franklin Lakes, NJ) in 24-well dishes (Nuncopore, Pleasanton, CA) were employed for invasion assays as described previously (14). Cell proliferation was measured by incorporation of [3H]thymidine as described previously (19).

Immunohistochemistry. Protein expression in cancer patients was detected by immunohistochemistry as described previously (18). A scoring system was devised to assign a staining intensity score for CNTN-1 expression from 0 (no expression) to 3 (highest intensity staining). Immunostaining was classified in the following two groups according to both intensity and extent: low expression, no staining was present (staining intensity score = 0), or positive staining was detected in <10% of the cells (staining intensity score = 1); high expression, positive immunostaining was present in 10% to 25% (staining intensity score = 2) or >25% of the cells.
results in a higher level above baseline. For a chosen threshold, a smaller starting copy number which the fluorescence generated by cleavage of the probe exceeds a fixed at a dilution of 1:50 and incubated at 4
paraformaldehyde and then blocked by incubation in 2.5% bovine serum Cells to be analyzed by immunofluorescent staining were fixed in 3%
was detected as a single yellow image when the images from both channels (488 nm) and HeNe (543 nm) lasers. Colocalization of two labeled antigens
invert, Carl Zeiss, Oberkochen, Germany), equipped with both argon ion
analyzed using an inverted laser scanning microscope (Zeiss LSM 410
fluorescence microscopy.

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(staining intensity score = 3). All of the immunohistochemical staining results were reviewed independently by two pathologists.

Real-time quantitative reverse transcription-PCR. The quality of RNA in samples was determined by electrophoresis through agarose gels and staining with ethidium bromide. 18S and 28S RNA bands were visualized with UV illumination. The primers used, based on the cDNA sequence of CNTN-1 (69 bp), were as follows: 5’-CAATAGTAGGGTGAGAC-3’ (forward), 5’-TGCGCTAGGCTTCTTTT-3’ (reverse), and 5’-CCAATGTGAGCTGTCCACC-3’ (probe). The probe was labeled at the 5’ end with carboxyfluorescein and at the 3’ end with AXN,N’-tetramethyl-6-
carboxyrhodamine. The primers and probe used for quantitative reverse transcription-PCR (RT-PCR) of the TATA box–binding protein mRNA (internal control, Genbank accession no. X54993) were as described previously (15). The threshold cycle (Ct) is the fractional cycle number at which the fluorescence generated by cleavage of the probe exceeds a fixed level above baseline. For a chosen threshold, a smaller starting copy number results in higher Ct values.

Immunofluorescence staining and laser scan confocal microscopy. Cells to be analyzed by immunofluorescent staining were fixed in 3% paraformaldehyde and then blocked by incubation in 2.5% bovine serum albumin in PBS. Primary antibodies as indicated were applied to the slides at a dilution of 1:50 and incubated at 4°C overnight. The samples were treated with FITC-conjugated or TRITC-conjugated secondary antibody (Sigma). The FITC-labeled or TRITC-labeled cells were then analyzed by fluorescence microscopy.

For laser scan microscopy, immunofluorescence-labeled cells were analyzed using an inverted laser scanning microscope (Zeiss LSM 410 invert, Carl Zeiss, Oberkochen, Germany), equipped with both argon ion (488 nm) and HeNe (543 nm) lasers. Colocalization of two labeled antigens was detected as a single yellow image when the images from both channels were overlaid. For each image, the cells were optically sectioned from the ventral to the dorsal surface at intervals of 1 μm.

Immunoprecipitation and Western blotting. The cellular lysates were prepared as described previously (18). Equal amounts of protein were incubated with specific antibody immobilized onto protein A-Sepharose beads. Solubilized proteins were analyzed by Western blotting as described previously (18). Where indicated, the membranes were stripped and reprobed with another antibody.

RNA isolation and RT-PCR. The RNA was prepared as described previously (18). Amplification of growth factor cDNAs and β-actin mRNA as internal controls in each reaction was done by the PCR with the following primer pairs: the primer sequences for CNTN-1 were 5’-TGTCAGCGAATCTCATTACC-3’ (forward) and 5’-CTACCACATCAAGGAAAGTGC-3’ (reverse) and for β-actin were 5’-GATGATGATATCGCCGCGCT-3’ (forward) and 5’-TGGTTTACATCTTGCAGGGT-3’ (reverse). Denaturation was done at 95°C for 10 minutes. The PCR conditions were 30 cycles of 95°C for 1 minute, 55°C for 1 minute, and 72°C for 1 minute, then 72°C for 10 minutes. PCR products were visualized by ethidium bromide staining after separation by agarose gel electrophoresis.

RNA interference and stably transfected clone selection. Three short interfering RNA (siRNA)–coding oligos against human CNTN-1 were designed and verified to be specific for CNTN-1 by a Blast search done against the human genome. The Contactin-1/siRNA-1–targeting sequence is CTACAGTGTTAGCAGAGTITA; the Contactin-1/siRNA-2–targeting sequence is ACAGAAAGATGCTGGAATATA; and the Contactin-1/siRNA-3–targeting sequence is GTAGTCAGCGAATCTCATTACC (reverse) and for β-actin were 5’-GATGATGATATCGCCGCGCT-3’ (forward) and 5’-TGGTTTACATCTTGCAGGGT-3’ (reverse). Denaturation was done at 95°C for 10 minutes. The PCR conditions were 30 cycles of 95°C for 1 minute, 55°C for 1 minute, and 72°C for 1 minute, then 72°C for 10 minutes. PCR products were visualized by ethidium bromide staining after separation by agarose gel electrophoresis.

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400 µg/mL hygromycin B. Hygromycin-resistant clones were selected, and these clones were then expanded for further studies.

Separation of nuclear and cytosolic fractions. Cytosolic and nuclear fractions were prepared as described previously (20).

Rho family pull-down assay. The Rho family pull-down assay was done as described previously (20).

Mice and measurement of s.c. tumor growth. Six-week-old severe combined immunodeficient (SCID) mice were inoculated s.c. with tumor cells (10^6 per animal). Tumor development was determined for individual animals (15 per group) by twice weekly sequential caliper measurements of length (L) and width (W). Tumor volume was calculated by the formula LW^2/2. After 10 weeks, the mice were sacrificed, and the tumors and lungs were removed and weighed. Segments of tumor and lung were excised and fixed in 10% neutral buffered formalin. All animal work was done according to protocols approved by the Institutional Animal Care and Use Committee of the College of Medicine, National Taiwan University.

Experimental metastasis assay. The experimental metastasis assay was done as described previously (14).

Patients and specimens. Sixty-five patients who underwent surgery for adenocarcinoma of the lung at the National Taiwan University Hospital were included in the study. None of the patients had received neoadjuvant chemotherapy or radiation therapy before surgery. Paraffin-embedded, formalin-fixed surgical specimens were collected for immunohistochemical staining. The histologic classification of adenocarcinoma lung cancer was determined as recommended by the WHO (21). Tumor size, local invasion, lymph node metastasis, and final disease stage were determined as described previously (14). The follow-up period lasted up to 110 months. Patients who died of postoperative complications within 30 days following their surgery were excluded from the survival analysis.

To identify genes associated with invasion, established human lung adenocarcinoma cell lines with different invasive activities (22) were examined by cDNA microarray and a colorimetric detection system (23). All experiments involving the cDNA microarrays were done in triplicate and under the high-stringency conditions reported previously (15). The expression of CNTN-1 mRNA was found to be positively associated with cell invasiveness (data not shown). To confirm the results of cDNA array screening, expressions of CNTN-1 mRNA and CNTN-1 protein were measured by reverse transcription-PCR (RT-PCR) and Western blotting, respectively. Expression of CNTN-1 mRNA and CNTN-1 protein was found to be elevated in highly invasive CL1-3 and CL1-5 lung cancer cells but expression was barely detectable in the poorly invasive parental CL1-0 cells (Fig. 1A, left). CNTN-1 expression and invasive capacity were also examined in other lung adenocarcinoma cell lines. A549 cells, which have strong invasive

Figure 2. Suppression of CNTN-1 expression by stable siRNA decreases invasive ability and rearrangements of the actin cytoskeleton and focal adhesion structures in lung adenocarcinoma cells. A, top, expression of CNTN-1 in CL1-5 (left) and A549 (right) cells stably expressing control/siRNA– or CNTN-1–targeted siRNA as determined by immunoblotting. Bottom, relative invasive abilities of CL1-5 (left) and A549 (right) cells stably expressing control/siRNA– or CNTN-1–targeted siRNA. To determine relative invasiveness, values were normalized to that of CL1-5/control or A549/control cells. Columns, means of three independent experiments; bars, upper 95% CI. Contactin-1/siRNA–transfected cells had significantly lower invasive activities than control/siRNA–transfected cells. *, P < 0.05, two-tailed Student’s t test. B, presence of well-formed F-actin–containing microfilament bundles in A549/control cells (top) but not in A549/siRNA-2 cells (bottom). Cells were fixed and stained with Hoechst 33258 to visualize nuclei (blue) and with a rhodamine-conjugated anti-phalloidin antibody to detect F-actin (red). Stained preparations were then examined by laser scan confocal microscopy. Arrows, F-actin–containing microfilament bundles. C and D, presence of focal adhesion structures in cells. Cells were fixed, stained with Hoechst 33258, and labeled with either anti-paxillin (C) or anti-vinculin (D) antibodies. Preparations were incubated with TRITC-conjugated secondary antibody and then examined by laser scan confocal microscopy. Arrows, focal adhesions. Bar, 15 µm.

Statistical analysis. The data are presented as the mean ± SD. The Student’s t test was used to compare data between two groups. Statistical analyses of clinicopathologic data were done as described previously (15). Survival curves were obtained using the Kaplan-Meier method. All statistical tests included two-way ANOVA. P ≤ 0.05 were considered to be statistically significant.

Results

CNTN-1 expression correlates with in vitro invasive ability and stage, lymph node metastasis and survival in lung cancer patients. To identify genes associated with invasion, established human lung adenocarcinoma cell lines with different invasive activities (22) were examined by cDNA microarray and a colorimetric detection system (23). All experiments involving the cDNA microarrays were done in triplicate and under the high-stringency conditions reported previously (15). The expression of CNTN-1 mRNA was found to be positively associated with cell invasiveness (data not shown). To confirm the results of cDNA array screening, expressions of CNTN-1 mRNA and CNTN-1 protein were measured by reverse transcription-PCR (RT-PCR) and Western blotting, respectively. Expression of CNTN-1 mRNA and CNTN-1 protein was found to be elevated in highly invasive CL1-3 and CL1-5 lung cancer cells but expression was barely detectable in the poorly invasive parental CL1-0 cells (Fig. 1A, left). CNTN-1 expression and invasive capacity were also examined in other lung adenocarcinoma cell lines. A549 cells, which have strong invasive
activity (Fig. 1A, right), expressed CNTN-1 more abundantly compared with H928 cells, which have low invasive activity (Fig. 1A, right). In addition, expression of CNTN-1 in normal lung epithelial cells was undetectable (data not shown). Expression of CNTN-1 was further determined in tumor samples from 65 lung adenocarcinoma patients by immunohistochemistry using an anti-CNTN-1–specific monoclonal antibody (MAB904) from R&D Systems. A moderate/strong staining for plasmalemmal CNTN-1 was frequently observed in advanced stage lung adenocarcinoma (Supplementary Fig. S1A); this positive staining was not obtained with preimmune serum or with antigen-blocked antibody (data not shown). In contrast, negative or very weak staining of CNTN-1 by MAB904 was seen in early-stage lung adenocarcinoma (Supplementary Fig. S1B). More importantly, strong staining of CNTN-1 was observed in metastatic tumor cells in lymph node sections (Supplementary Fig. S1C); this positive staining was not observed with preimmune serum or with antigen-blocked antibody (data not shown). In contrast, negative or very weak staining of CNTN-1 by MAB904 was seen in early-stage lung adenocarcinoma (Supplementary Fig. S1A). This positive staining was not obtained with preimmune serum or with antigen-blocked antibody (data not shown).

Figure 3. CNTN-1 is colocalized with RhoA and required for RhoA activation. A, expression of various Rho family GTPases in A549 cells and CL1-5 cells transfected with contactin-1/sirna-2 or control-sirna by Western blot assay and GST pull-down assay. Findings were repeated on three separate occasions, and representative findings are presented. B, membrane and cytosolic fractions were derived from lysates, and solubilized proteins from each fraction were subjected to SDS-PAGE and immunoblotting for RhoA and Rac-1. Findings were repeated on three separate occasions, and representative findings are shown. C, A549 cells were treated with the indicated concentrations of CNTN-1–neutralizing antibody for 24 or 48 hours. Activation of RhoA (top) and invasive ability (bottom) were measured. CNTN-1–neutralizing antibody-treated cells had significantly lower invasive activities than normal IgG-treated cells. *, P < 0.05, two-tailed Student’s t test. Columns, means of three independent experiments; bars, upper 95% CI. D, top, association of membrane-localized CNTN-1 with RhoA in lung cancer cells. Colocalization of two labeled antigens was detected as a single yellow image when the images from both channels were overlaid. Bottom, membrane fractions prepared from A549 and CL1-5 cells transfected with contactin-1/sirna-2 or control/sirna were incubated with anti-RhoA antibody. Immunoprecipitated proteins were collected and subjected to SDS-PAGE and immunoblotting with anti-CNTN-1 or anti-RhoA antibody. Representative of three independent experiments.

The surgical-pathology stages of the 65 patients described above were stage I for 26 patients, stage II for 10 patients, stage III for 24 patients, and stage IV for 5 patients. Tumor status was T1 for 18 patients, T2 for 36 patients, T3 for 3 patients, and T4 for 8 patients. Thirty patients had no lymph node metastasis (N0), and 35 patients had regional or mediastinal lymph node metastases (N1 in 13 patients, N2 in 21 patients, and N3 in 1 patient). Immunohistochemical analysis of the 65 tumor specimens revealed high CNTN-1 expression for 58.5% (38 of 65) of the lung adenocarcinoma patients. CNTN-1 high-expression patients were more likely than low-expression patients to have advanced disease (stage III or IV; P < 0.001), advanced tumor status (T2, T3, and T4; P = 0.048), and lymph node metastasis (N1, N2, and N3; P < 0.001). The median duration to postoperative recurrence was also longer in CNTN-1 low-expression patients (57.9 months) than in CNTN-1 high-expression patients (57.9 months). The probability of survival reached a plateau value of 0.54. Their survival was increased in a statistically significant manner when compared with CNTN-1 high-expression patients (median survival = 19.4 ± 4.5 months; 95% CI, 10.7-28.2 months; log-rank test, P < 0.001; Fig. 1C).

CNTN-1 is essential for invasion and for rearrangements of the actin cytoskeleton and focal adhesion structures in lung adenocarcinoma cells. To ascertain whether CNTN-1 plays a
critical role in tumor metastasis, three human CNTN-1–targeted RNA interference expression vectors (siRNA) or a control vector was transfected into highly metastatic lung adenocarcinoma cells. As shown in Fig. 2A, expression of each of the three siRNAs, but not of the control siRNA, dramatically reduced the expression of CNTN-1 protein (Fig. 2A, top). The contactin-1/siRNA-2 proved especially effective, suppressing the expression of CNTN-1 protein in both cell lines to degrees that were undetectable by immunoblotting (Fig. 2A, top). The expression of contactin-1/siRNA-2 in CL1-5 cells and A549 cells also strongly impaired their invasive activities (Fig. 2A, bottom) but not proliferation rates (data not shown). These data rule out the possibility that the effects of CNTN-1 on in vitro cell invasiveness were attributable to the different proliferation rates.

F-actin is continuously polymerized and depolymerized in motile cells and is essential to cell motility (24). To further assess the role of CNTN-1 arrangements of the actin cytoskeleton and focal adhesion structures, A549/siRNA-2 and A549/control cells were, therefore, stained with rhodamine-conjugated anti-phalloidin antibody and characterized using multicolor immunofluorescence and confocal laser scanning microscopy. A549/control cells displayed well-formed F-actin–containing microfilament bundles within the cytoplasm and below the plasma membrane (Fig. 2B, top). However, cells expressing contactin-1/siRNA-2 contained few, if any, microfilament bundles (Fig. 2B, bottom). A549/control cells displayed a large number of prominent paxillin-containing (Fig. 2C, top) and vinculin-containing (Fig. 2D, top) focal adhesions at the cell periphery. Stable expression of contactin-1/siRNA-2 resulted in a decrease in focal adhesion formation as assessed by paxillin and vinculin staining (Fig. 2C and D). These findings strongly support the hypothesis that CNTN-1 is intimately involved in regulation of the invasive activity of lung adenocarcinoma cells.

**RhoA is required for CNTN-1–mediated cell invasion.** The Rho family of small GTPases has recently been implicated in the invasive and metastatic capacities of various cancer cells (25, 26). It was, therefore, of interest to explore the possibility that GTP-binding proteins of the Rho family were involved in CNTN-1–mediated invasion of lung adenocarcinoma cells. As shown in Fig. 3A, expression of contactin-1/siRNA-2 decreased the activity of RhoA (relative amount of RhoA in the active form) but not of the two other Rho family small GTPases, Rac1 and Cdc42, in both A549 and CL1-5 cells. The activities of these GTPases were not altered in cells transfected with control vector. The activity of RhoA was either very low or undetectable in the less invasive and low CNTN-1–expressing CL1-0 and H928 cells (data not shown). In addition to the findings from glutathione S-transferase (GST) pull-down assays, expression of contactin-1/siRNA-2 was found to promote the redistribution of RhoA but not of Rac1 from the membrane to the cytosol (Fig. 3B). The expression of active RhoA (RhoA/GTP) and in subsequent invasive ability were concentration-dependently decreased by CNTN-1 neutralizing antibody but not with control antibody (Fig. 3C).

Members of several classes of adhesion molecules, including integrins, cadherins and immunoglobulin superfamily members, are reported to form complexes with Rho family proteins, thereby altering their activities (27). Multicolor immunofluorescence staining and confocal laser scanning microscopy were, therefore, employed to ascertain whether membrane-specific RhoA associates with CNTN-1 in A549/control and A549/siRNA-2 cells. Plasmalemmal RhoA was expressed more abundantly in A549/control cells and was colocalized with CNTN-1 (Fig. 3D, top). In contrast, A549 cells expressing contactin-1/siRNA-2 displayed decreased CNTN-1 expression and a loss of associations between CNTN-1 and membrane-localized RhoA (Fig. 3D, top). In a separate experiment, expression of contactin-1/siRNA-2 dramatically decreased the association of RhoA with CNTN-1 in membrane fractions prepared from both A549 and CL1-5 cells (Fig. 3D, bottom). Transfection with the constitutively activated RhoA (V14RhoA) expression vector resulted in increased expression of activated RhoA and slight enhancement of invasive activity in A549/control cells (Fig. 4A).

**Figure 4.** RhoA activity is required for CNTN-1–mediated lung adenocarcinoma cell invasion and F-actin polymerization. A, activation of RhoA and invasive activity of A549/control and A549/siRNA-2 cells as a function of stable transfection with the constitutively activated RhoA (pcDNA3.1-V14RhoA) expressing vector. Top, activation of RhoA was measured by the GTP pull-down assay. Bottom, invasive activity was measured by an in vitro invasion assay with values expressed as percentage of the untreated control. Bars, upper 95% CI. pcDNA3.1-transfected A549/siRNA-2 cells had significantly lower invasive activities than pcDNA3.1-transfected A549/control cells. *, P < 0.05, two-tailed Student’s t test. Transfection of A549/siRNA-2 cells with pcDNA3.1-V14RhoA restored invasive activity significantly compared with pcDNA3.1-transfected A549/siRNA-2 control cells. #, P < 0.05, two-tailed Student’s t test. B, formation of F-actin filaments in cells stably transfected with pcDNA3.1-V14RhoA or pcDNA3.1. Arrows, F-actin–containing microfilament bundles. C, expression of CNTN-1, total RhoA, and activated RhoA in tissue specimens from lung adenocarcinoma patients with (lanes 1–8) or without (lanes 9–16) lymph node metastasis by Western blotting and GST pull-down assay.
Furthermore, the observed contactin-1/siRNA-2–dependent reductions of RhoA activation and invasive ability described above were both significantly overturned by transfection with V14RhoA expression vector (Fig. 4A). In addition, the decrease in F-actin–containing microfilament bundles resulting from expression of contactin-1/siRNA-2 was also reversed by transfection with the V14RhoA expression vector but not with a control vector (Fig. 4B). These findings are consistent with a critical role for activated RhoA in adenocarcinoma cell invasion and in CNTN-1–dependent F-actin polymerization in these cells.

To explore the relationships among CNTN-1 expression, RhoA activity, and metastatic potential in lung adenocarcinoma tumors, measurements of CNTN-1 expression and RhoA activity were done for specimens from lung adenocarcinoma patients with or without metastasis. As shown in Fig. 4C, CNTN-1 protein was clearly expressed in specimens from patients with metastatic lung adenocarcinoma but was only modestly expressed or undetectable in specimens from lung adenocarcinoma patients without metastasis. Furthermore, the activity of RhoA was significantly higher in specimens from patients with metastasis (Fig. 4C). These correlative findings support relationships between RhoA activity and CNTN-1 expression in human lung adenocarcinoma tumors.

CNTN-1 is essential for tumor metastasis. To determine whether CNTN-1 plays a causal role in tumor metastasis, A549/control, A549/siRNA-2, CL1-5/control, and CL1-5/siRNA-2 cells were injected s.c. into the right flank of SCID mice followed by measurements of growth of the resulting primary tumors at five day intervals. A549 cells and CL1-5 cells carrying either contactin-1/siRNA-2 or the control siRNA formed primary tumors at identical rates (Fig. 5A), indicating that high expression of CNTN-1 is not required for primary tumor formation by these cells. To determine whether loss of CNTN-1 expression affected the ability of A549 cells or CL1-5 cells to metastasize, the metastatic behaviors of the A549 and CL1-5 tumor cells expressing either contactin-1/siRNA-2 or the control siRNA vector were compared. Although cells expressing control siRNA formed large numbers of macroscopically visible metastases in lungs, cells that expressed contactin-1/siRNA-2 formed very few metastases (Supplementary Fig. S2). A mean of 44 metastatic lung nodules was obtained for mice injected with A549/control cells, whereas a mean of 5 metastatic lung nodules was obtained for mice injected with A549/siRNA-2 cells (difference = 39 nodules; 95% CI, 28-49 nodules; P < 0.001). A mean value of 38 metastatic lung nodules was obtained for mice injected with CL1-5/control cells, whereas the mean value for
mice injected with CL1-5/siRNA-2 cells was 5 metastatic lung nodules (difference = 33 nodules, 95% CI 26 to 41 nodules; \( P < 0.001 \)). Suppression of CNTN-1 expression in CL1-5 and A549 cells was therefore concluded to decrease the ability of these cells to form metastatic nodules in the lungs. The mean weight of lungs from mice injected with A549/siRNA-2 cells was 440 mg, whereas that from mice injected with A549/control vector control cells was 552 mg (difference = 112 mg; 95% CI of the difference, 77-147 mg; \( P < 0.001 \)). The mean weight of lungs from mice injected with CL1-5/siRNA-2 cells was 440 mg, whereas that from mice injected with CL1-5/control vector control cells was 502 mg (difference = 62 mg; 95% CI of the difference, 26-97 mg; \( P = 0.001 \)). Mean lung weight was, therefore, decreased by 20.3% in mice injected with A549/siRNA-2 cells and by 12.4% in mice injected with CL1-5/siRNA-2 cells compared with lung weights in mice injected with vector control cells. Data from these two experiments are summarized in Table 1.

Metastasis is believed to represent the factor most critical to mortality of cancer patients (1). As shown in Fig. 5B, the survival time of mice injected with A549/siRNA-2 cells was 59.27 days, and was significantly longer than that of mice injected with A549/control cells (43.87 days; \( P < 0.001 \), log-rank test). Consistent with these findings, the survival time of mice injected with CL1-5/siRNA-2 cells was 64.93 days, and was significantly longer than that of mice injected with CL1-5/control cells (53.60 days; \( P < 0.05 \), log-rank test). Suppression of CNTN-1 was therefore concluded to decrease the metastatic ability of lung adenocarcinoma cells in vivo and to promote the survival of tumor-bearing mice. The primary tumors formed from A549/control, A549/siRNA-2, CL1-5/control, or CL1-5/siRNA-2 cells were collected, subjected to homogenization, and analyzed for expression of CNTN-1 protein and for RhoA activity. The expression of CNTN-1 and the activity of RhoA in primary tumors formed from A549/siRNA-2 and CL1-5/siRNA-2 cells were significantly higher than tumors from A549/control or CL1-5/control cells (Fig. 5C).

To ascertain whether RhoA is critical for CNTN-1–mediated metastatic colonization, A549/control cells and A549/siRNA-2 cells were each stably transfected with V14RhoA expression vector or control vector (A549/control/vector, A549/control/V14RhoA, A549/siRNA-2/vector, or A549/siRNA-2/V14RhoA) and evaluated in an in vivo experimental metastasis assay. Mice injected with A549/siRNA-2/vector cells exhibited a lower occurrence of lung metastases than did those injected with A549/control/vector cells. This decreased occurrence in vivo was overturned significantly by expression of V14RhoA in the A549/siRNA-2 cells (50% occurrence for A549/siRNA-2/vector cells versus 87.5% occurrence for A549/siRNA-2/V14RhoA cells; Fig. 5D). The average numbers of visible metastatic nodules in mice injected with A549/control/vector and A549/control/V14RhoA cells were 85.38 ± 31.42 and 99.88 ± 22.86 (mean ± SD), respectively. As shown in Fig. 5D, the average number of metastatic nodules in mice injected with A549/siRNA-2/vector was dramatically reduced compared with A549/control/vector cells (85.38 ± 31.42 for A549/control/vector cells versus 10.75 ± 10.59 for A549/siRNA-2/vector cells; \( P < 0.001 \)). This reduction in number of metastatic nodules could be diminished significantly by transfection with the V14RhoA expression vector (10.75 ± 10.59 for A549/siRNA-2/vector cells versus 51.25 ± 36.66 for A549/siRNA-2/V14RhoA cells; \( P = 0.01 \)). Taken together, the above findings strongly support a requirement for RhoA in CNTN-1–mediated metastasis of lung adenocarcinoma cells in vivo.

### Discussion

By using genome-wide cDNA microarray screening for invasion-associated genes, two potent metastasis suppressors were identified previously in this laboratory (14, 15). The present study, which also used the screening approach, provides evidence that CNTN-1 is essential for the invasion and metastasis of human lung adenocarcinoma. CNTN-1 expression was shown to be associated with clinical metastasis and patient survival, and reduced CNTN-1 expression was found to be associated with the suppression of human lung cancer cell metastasis in a mouse model. Furthermore, one of the Rho family GTPases, RhoA, was found to be required for CNTN-1–mediated invasion and metastasis of human lung adenocarcinoma. These findings not only serve to delineate a function for CNTN-1 in lung adenocarcinoma but also provide new information regarding the mechanisms through which this type of cancer becomes metastatic.

Human CNTN-1 is a member of the contactin subgroup of the immunoglobulin superfamily. Other members of this family include contactin-2 (TAG-1), contactin-5 (NB-2), and contactin-6 (NB-3). The best-characterized function of these proteins is the repulsive guidance of nerve axons, the molecular mechanism of which remains to be characterized (28). CNTN-1 is a glycosyl phosphatidylinositol anchor neural cell adhesion molecule (NCAM) differentially expressed in numerous neuronal tissues and thought to function in nervous system development (29). CNTN-1 has been observed to associate with other cell surface proteins believed to participate in various signal transduction pathways and cell functions. CNTN-1 interacts in trans with the \( \beta \)-isoform of receptor protein tyrosine phosphatase (RPTP) to promote neurite outgrowth (30) and in cis with the \( \alpha \)-isoform of RPTP (31) to transduce extracellular signals to Fyn kinase (32), a member of the Src kinase family.

### Table 1. Suppression of metastasis by RNA interference of CNTN-1 expression in lung adenocarcinoma cells

<table>
<thead>
<tr>
<th>Cell lines</th>
<th>Lung weight</th>
<th>Lung metastasis</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Mean ± SD (mg)</td>
<td>P</td>
</tr>
<tr>
<td>A549/control</td>
<td>552 ± 51</td>
<td></td>
</tr>
<tr>
<td>A549/siCNTN-1</td>
<td>440 ± 42</td>
<td>&lt;0.001*</td>
</tr>
<tr>
<td>CL1-5/control</td>
<td>502 ± 52</td>
<td></td>
</tr>
<tr>
<td>CL1-5/siCNTN-1</td>
<td>440 ± 41</td>
<td>0.001*</td>
</tr>
</tbody>
</table>

*A549/control vs A549/siCNTN-1; CL1-5/control vs CL1-5/siCNTN-1 (Student’s \( t \) test).
family that regulates cell mobility (33). The CNTN-1–associated protein RPTPβ has been reported to bind to a variety of cell adhesion molecules and components of the extracellular matrix, such as NCAM and pleiotrophin (34). Interestingly, both RPTPβ and RPTPβ interact with and regulate the tyrosine phosphorylation of catenins, proteins that are critical to physiologic and pathologic processes, such as cell migration, adhesion, and transformation (34). The present study shows, for the first time, that the small GTPase called RhoA is a novel CNTN-1–associated protein essential to CNTN-1–mediated invasion and metastasis of lung adenocarcinoma cells (Figs. 3 and 4). Whether the CNTN-1/RhoA complex also associates with other cell mobility–related proteins, such as members of the Src kinase family or isoforms of RPTP, requires further investigation.

Tumor metastasis could result from decreased expression of tumor suppressor genes, such as NM23, CRMP-1, and CTGF (14, 15, 35), and/or increased expression of metastasis-promoting genes, such as CNTN-1. To ascertain whether suppression of CNTN-1 increases the expression of potent suppressors of metastasis, the expression of NM23, CRMP-1, and CTGF proteins in contactin-1/siRNA-2 stably transfected A549 and CL1-5 cells was measured. Stable expression of contactin-1/siRNA-2 did not affect nm23, CRMP-1 or CTGF protein expression in either A549 or CL1-5 cells (4). Two in vivo models were used in this study: one is s.c. transplanted cancer cells in mice, another is i.v. injection of cancer cells into lateral tail vein of mice. Our data indicated that CNTN-1 plays a critical role in metastasis of lung adenocarcinoma cells through RhoA-dependent pathway. Otherwise, the animal models we used in this study are not physiologic metastatic model, and this is a weakness in our study. The surgical orthotopic implantation is the more clinic-like metastasis model and may provide additional insights (36).

Rho proteins, including Cdc42, Rac1, and RhoA, are best characterized for their effects on the cytoskeleton and cell adhesion. Because cell mobility is likely to be influenced by these effects, invasion and metastasis are also likely to be affected (37). During stimulated migration of NIH3T3 cells, Cdc42 is activated and induces formation of thin protrusions rich in actin and known as microspikes or filopodia (38). Activation of Rac1 promotes formation of sheath-like protrusions termed lamellipodia to promote an increase in cell mobility (39–41). In addition to regulation of protrusive events, Cdc42 and Rac1 both promote formation of the smaller focal complexes (39). Activation of RhoA is recognized to mediate formation of stress fibers and focal contacts that firmly anchor cells to their substrata, permitting retraction of cellular rear ends (42, 43). In the present study, RhoA was found to be required for CNTN-1–mediated F-actin bundle formation, cell invasion, and in vivo metastasis of lung adenocarcinoma cells. Other investigators reported recently (25–27) that Rho-related GTPases can also regulate gene expression, often through the activation of kinase cascades leading to enhanced activity of stress-activated protein kinases, such as c-Jun NH2-terminal kinase and p38 mitogen-activated protein kinase, to alter cell survival. In the present study, however, CNTN-1–mediated RhoA activation was observed to be essential to cell invasion but not to cell proliferation in an animal model (Fig. 4A).

Vinculin plays a central role in the mechanical coupling of integrins to the cytoskeleton as well as in the control of cytoskeletal mechanics, cell shape, and motility (44, 45). Paxillin, which can interact with a variety of signaling proteins, is presumed to be involved in several signaling pathways as well as in anchoring of the actin cytoskeleton (46). In the present study, knockdown of CNTN-1 expression resulted in significant changes in the distribution of vinculin and paxillin concurrent with suppression of cell invasion (Fig. 2C and D). Additional studies to ascertain whether selected integrins or other signaling proteins are involved in CNTN-1–induced macroaggregation of vinculin and paxillin in lung adenocarcinoma cells should prove helpful in characterizing further the mechanisms underlying invasion by these cells.

In the studies described in the present report, suppression of CNTN-1 was shown not only to suppress the ability of lung adenocarcinoma cells to invade Matrigel in vitro but also to inhibit tumor metastasis strongly in an animal model. Regarding the mechanisms through which CNTN-1 affects tumor invasion, RhoA was observed to interact with CNTN-1 at the cell membrane and to play a critical role in CNTN-1–mediated invasion and metastasis. CNTN-1 was differentially expressed in tumor tissues, and its expression was directly correlated with tumor stage, lymph node metastasis, and patient survival. These findings are fully consistent with involvement of CNTN-1 in the invasion and metastasis of lung adenocarcinoma cells. Further investigations are nonetheless required to characterize in full the mechanism(s) through which CNTN-1 modulates carcinoma cell invasion and metastasis.

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References


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