A kelch family protein Nd1-L functions as a metastasis suppressor in cancer cells via Rho family proteins mediated mechanism

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Abstract. The BTB-kelch protein Nd1-L acts as an actin cytoskeleton stabilizer expressed ubiquitously in mouse tissues. We examined the effect of Nd1-L on cancer cell invasion and metastasis. Over-expression of Nd1-L in murine colon carcinoma cell line Colon 26 and murine melanoma cell line B16 resulted in suppression of pulmonary and liver metastasis after inoculation of these cells to syngeneric mice and in increased survival in an animal model. On the other hand, knock down of Nd1-L by RNA interference promoted metastasis ability of these cells. Increased expression of Nd1-L inhibited migration and Matrigel invasion capacity of cancer cell lines *in vitro*. Thus, Nd1-L expression inversely correlated with invasive and metastasis capacity of cancer cells. Furthermore, increased expression of Nd1-L in NIH3T3 cells inhibited growth factor induced activation of Rho family small GTPases such as Rho, Rac and cdc42. These results suggest that Nd1-L is involved in invasion and metastasis of cancer cells by regulating the actin cytoskeleton and Rho family proteins.

Introduction

The kelch family proteins contain 4-7 kelch motifs that form a propeller-like structure (1). In addition to the kelch motifs, the majority of the kelch proteins contain a BTB/POZ domain (broad complex, tramtrack and brick a brac/Pox virus and zinc finger) that mediates protein-protein interactions (2). Bioinformatics analysis identified at least 71 kelch proteins in the human and mouse genome, which reveals that the kelch motif is widely distributed (3). Many kelch domain-containing proteins interact with actin and are important mediators of fundamental cellular functions, such as regulation of cellular architecture, cellular organization, and cell migration (4-6). More recently, it has been suggested that the kelch repeats may also bind to proteins other than actin. It has also been shown that some BTB-kelch proteins serve as a substrate-specific adaptor for cullin 3 ubiquitin ligases (7-9). Nd1-L contains a BTB/POZ domain in its N terminus and six kelch repeats in the C terminus (10). In NIH3T3 cells, Nd1-L co-localized with actin filaments in stress fibers and stabilized actin cytoskeleton (10). Overexpression of Nd1-L in the heart of transgenic mice protected the cardiomyocyte apoptosis and improved a survival rate after doxorubicin injection (11). Furthermore, study of Nd1-L knockout mice revealed that Nd1-L plays a protective role in doxorubicin-induced cardiotoxic responses (12).

It has been shown that some BTB-kelch proteins play important roles in cancer. For example, point mutations of Keap1, which induce loss of its function, exist in lung cancer (13). Mutations in the neuronal nuclear matrix protein NRPB/ENC1 were observed in brain tumors (14,15). In addition, the kelch motif-containing protein Mayven was abundant and diffuse in primary human epithelial breast tumor cells as compared to normal breast epithelial cells, where Mayven was detected in the normal breast layer of the mammary ducts (16). Metastasis is the most life-threatening complication of cancer and major problem of cancer treatment. Tumor cell migration and invasion are essential steps for establishment of cancer metastasis (17). It is well known that the actin cytoskeleton is a major component of the cell motility machinery. Changes in actin cytoskeleton organization, adhesiveness and motility are important not only for tumor development and progression but also may be critical for determining invasion and metastasis potential of tumor cells (18,19). However, the molecular mechanisms underlying the invasion and metastasis of tumor cells are not well understood. Genome-wide screening of genes responsible for virulence of cancer cells or metastatic ability has been performed using primary cancer cells or cancer cell lines (20-22). Genes encoding actin-binding protein, matrix metalloproteinase, chemokine

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and chemokine receptors or adhesion molecules were listed as candidate genes (22).

In this study, we examined the effect of Nd1-L on invasion, metastasis and virulence of cancer cells using murine colon carcinoma cells and murine melanoma cells. Here we show that Nd1-L functions as a metastasis suppressor in *in vivo* animal model. Amount of Nd1-L in these cancer cells inversely correlated with invasion and metastatic ability *in vitro* and *in vivo*. Furthermore, Rho family small GTPases activity was inhibited by over-expression of Nd1-L in NIH 3T3 cells. We also discuss the role of Nd1-L in cancer cell invasion and metastasis.

Materials and methods

Cell culture. NIH3T3 cells and B16 murine melanoma cells were maintained in Dulbecco's modified Eagle's medium (Sigma) supplemented with 10% fetal bovine serum (Sigma) at 37˚C, 5% CO_2. Colon 26 murine colon adenocarcinoma cells were maintained in RPMI (Sigma) supplemented with 10% fetal bovine serum (Sigma) (23). In some experiments, lysophosphatidic acid (LPA) (Sigma) or PDGF-BB (Pepro Tech Inc.) was added in the culture medium.

Construction of FLAG epitope-tagged Nd1-L expression plasmid (pCR-2FLAG-Nd1-L). Flag-Nd1-L expression plasmids (pCR-2FLAG-Nd1-L) were constructed by PCR amplifying Nd1-L fragments containing an open reading frame and ligating them to *Eco*RI site of pCR-2FLAG (10).

Construction of Nd1-L RNA interference plasmid (pBAsi-Nd1-L). A short interfering RNA (siRNA)-cording oligo against Kelch motif of Nd1-L was designed and verified to be specific for Nd1-L by a Blast search done against the mouse genome. The Nd1-L/siRNA-targeting sequence is 5'-GTCTACAACCCTCAGTCAA-3'. The U6 promoter with Nd1-L/siRNA was cloned into a commercially available vector pBAsi-mU6 Neo (Takara) to make pBAsi-Nd1-L. A siRNA oligo used as a negative control is 5'-GACTTCGTCA CGTAGTAAC-3'.

Transfection. For transfection, NIH3T3 cells ($4.5x10^5$ cells/60 mm dish or $1.3x10^6$ cells/10 cm dish), Colon 26 cells ($4.0x10^5$ cells/60 mm dish or $1.2x10^6$ cells/10 cm dish), and B16 cells ($6.0x10^5$ cells/60 mm dish or $1.8x10^6$ cells/10 cm dish) were plated 24 h before transfection. Cells were transfected with 3.0 μg/60 mm dish or 15 μg/10 cm dish of pCR3.1-2FLAG-Nd1-L or pBAsi-Nd1-L using TransFast™ Transfection Reagent (Promega) and harvested after 48 h. To establish stable transfectants, NIH3T3 cells and Colon 26 cells were selected with 500 μg/ml G418 and B16 cells with 800 μg/ml G418.

Experimental metastasis. C57BL/6 Cr.Slc and BALB/c mice were purchased from Japan SLC Co. (Hamamatsu, Japan). The care of all animals used in the present study was in accordance with Chiba University Animal Care guidelines.

B16 cells were briefly trypsinized and suspended in PBS. Cells ($1.0x10^6$) were injected into the lateral tail vein of 6-7-week old female C57BL/6 mice in a volume of 0.2 ml. At 2 weeks after i.v. injection, mice were sacrificed and the lungs were extirpated and the black spherical B16 metastatic nodules were counted.

Colon 26 cells were briefly trypsinized and suspended in PBS. Cells ($1.0x10^5$) were injected into the tail veins of 6-7-week old female BALB/c mice. At 2 weeks after i.v. injection, mice were sacrificed and the lungs were extirpated and white Colon 26 metastatic nodules were counted. In order to reveal white metastatic nodules markedly, indocyanine green dye (ICG) (2.5 mg/ml) was injected into trachea in a volume of 1.0 ml to stain the lungs.

For liver metastasis experiments, cells were injected into the spleen of the mice and splenectomies were performed 5 min after intrasplenic injection. Ten days after inoculation, mice were sacrificed and liver metastasis were analyzed by measuring liver weights as described previously (24).

Measurement of primary tumor growth. Cells were harvested by trypsinization, suspended in PBS and counted. Cells ($1.0x10^6$) were injected subcutaneously at the abdomen in a volume of 0.1 ml. Primary tumor outgrowth was monitored at two-day intervals by taking measurements of the tumor length (L) and width (W). Tumor volume was calculated as $\pi LW^2/6$.

Spontaneous lung metastasis assay. B16 cells were harvested by trypsinization, suspended in PBS and counted. Cells ($1.0x10^6$) were injected subcutaneously at the back of anesthetized mice [Nembutal (pentobarbital sodium) 30 mg/kg] in a volume of 0.1 ml. Primary tumor outgrowth was monitored every 2 days by taking measurements of the tumor length (L) and width (W). Tumor volume was calculated as $\pi LW^2/6$. For spontaneous metastasis assays, tumors were surgically resected when they reached >500 mm^3. Three weeks after resection, the mice were sacrificed and their lungs were excised. The numbers of black lung metastatic nodules were counted under a dissecting microscope.

Invasion assay. Modified Boyden chambers with filter inserts (pore size of 8 μm) coated with Matrigel (BD Bioscience) in 24-well dishes were employed for invasion assays. NIH3T3 conditioned medium was added to the lower compartment of the chamber as the chemoattractant. Cells starved in DMEM containing 0.1% bovine serum albumin (DMEM-BSA) overnight were trypsinized briefly and collected, and then 500 μl cell suspension ($2x10^5$ cells/ml in DMEM-BSA) was added to upper compartment of the chamber and incubated at 37˚C in a humidified atmosphere with 5% CO_2 for 24 h. Cells on the upper surface of the membrane were removed with a cotton swab, and cells had migrated to the under surface of the membrane were fixed and stained with Diff-Quik (Sysmex International Reagents Co.) and counted phase-contrast microscope with a x20 objective lens. Numbers of cells in twelve randomly selected fields from triplicate chambers were counted.

Anti-Nd1-L antibody. Three rabbits were immunized with purified mouse Nd1-L produced in *E. coli* in Freund's complete adjuvant subcutaneously in their backs. After 2 boosts with purified Nd1-L in incomplete adjuvant, rabbits were bled.

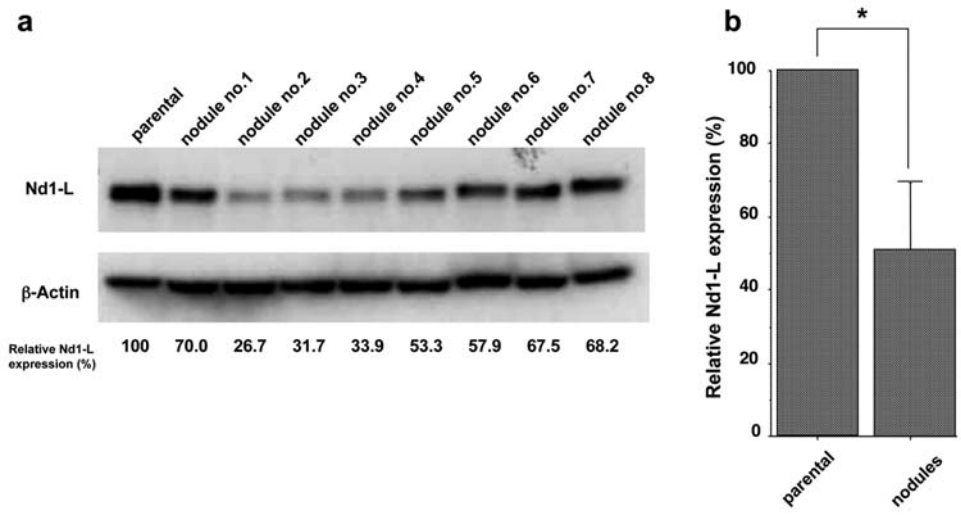

Figure 1. Reduction of Nd1-L expression in metastatic lesion of colon cancer cells in mice. (a) Colon 26 murine colon cancer cells were injected intravenously into syngeneic Balb/c mice. Cells derived from lung metastatic nodules (nos. 1-8) were analyzed for expression of Nd1-L protein by Western blot analysis. The parental cells are shown for reference. Relative expression of Nd1-L normalized by ß-actin was determined by densitometric analysis. (b) Average of relative Nd1-L expression in parental Colon 26 cells and lung metastatic nodules. Error bars represent mean ± SDs. *P<0.05.

Production of anti-mouse Nd1-L sera was checked by Western blot analysis using pre-immune sera as a negative control.

Western blotting. Cells were sonicated in lysis buffer (50 mM Tris-HCl, 150 mM NaCl, 1% Triton-X, 1 mM EDTA, 1 mM sodium orthovanadate, 1 mM phenylmethylsulfonyl fluoride, 10 μg/ml aprotinin, and 10 μg/ml leupeptin). Proteins were separated by SDS-PAGE and were transferred to poly-vinylidene difluoride membranes (Millipore). Membranes were blocked with non-fat dry milk (Yukijirushi) and were incubated with the following antibodies: mouse anti-Rho, mouse anti-Rac, mouse anti-Cdc42 (Upstate) or rabbit anti-Nd1-L antibody, followed by incubating with anti-rabbit (Jackson Immuno-Research Laboratories, West Grove, PA) or anti-mouse immunoglobulin antibody (Upstate). The filters were washed with TBS/0.5% Tween-20 and immuno-reactive bands were visualized with ECL system (Amersham Pharmacia). Band images were scanned and densitometric analyses were performed using NIH Image, version 1.63.

Rho family pull-down assay. The Rho family pull-down assay was performed as described previously (25,26). Briefly, cells were plated in 10-cm dishes, serum-starved for 12 h, and lysed in 25 mM HEPES, pH 7.5, 150 mM NaCl, 1% NP40, 10 mM $MgCl_2$, 1 mM EDTA, 1 mM sodium orthovanadate, 10 μg/ml leupeptin and 10 μg/ml aprotinin. One-tenth of the cell lysates were subjected to immunoblotting. The remaining cell lysate was mixed with 25 μg of bacterially expressed GST-rhotekin (murine amino acids 7-89, for RhoA-GTP) and 25 μg of bacterially expressed GST-PAK (human PAK-1 amino acids 67-150, for Rac1-GTP and Cdc42-GTP) bound to glutathione agarose (Upstate) and incubated at 4˚C with tumbling for 45-60 min. Beads were collected by centrifugation and washed three-times in 25 mM HEPES, pH 7.5, 150 mM NaCl, 1% NP40, 10 mM $MgCl_2$, 1 mM EDTA, 1 mM sodium orthovanadate, 10 μg/ml leupeptin and 10 μg/ml aprotinin before addition of Laemmli buffer and analyzed by Western blotting with anti-RhoA clone55, anti-Rac1 and anti-Cdc42 antibodies (Upstate).

Statistical analysis. The data are presented as the mean ± SD. Fisher's exact probability test was used to compare data between two groups. Survival curves were obtained using the Kaplan-Meier method. All statistical analyses were carried out using the StatView 5.0 J program for Windows (SAS Institute, Cary, NC). P-values <0.05 were considered statistically significant.

Results

Reduction of Nd1-L expression in metastatic tumor nodules. In order to examine whether Nd1-L is involved in tumor metastasis, we used murine metastatic colon tumor cell line Colon 26 to compare the expression of Nd1-L between parental cells and metastatic nodules. Two weeks after intravenous injection of the parental Colon 26 cells, metastatic nodules in lungs were isolated and expanded *in vitro* to examine the Nd1-L expression. Expression of Nd1-L protein reduced in all metastatic nodules from lungs compared to that of parental cells (Fig. 1). This result suggests that Nd1-L is one of the key determinants of whether a tumor cell will be more or less invasive and metastatic.

Nd1-L expression correlates with in vivo metastatic ability in murine cancer cell lines. Reduced Nd1-L expression was observed in a high percentage of Colon 26 metastatic nodules compared with parental cells, suggesting that it may contribute to the metastatic process. To investigate whether Nd1-L plays a critical role in tumor metastasis, Nd1-L expression vector (pCR-2FL-Nd1-L) or Nd1-L targeted RNA interference expression vector (pBAsi-Nd1-L) was transfected into Colon 26 and B16 cell lines. As shown in Fig. 2, Nd1-L siRNA suppressed the expression of endogenous Nd1-L protein in both Colon 26 cells (Fig. 2a; CKD1 and CKD2)

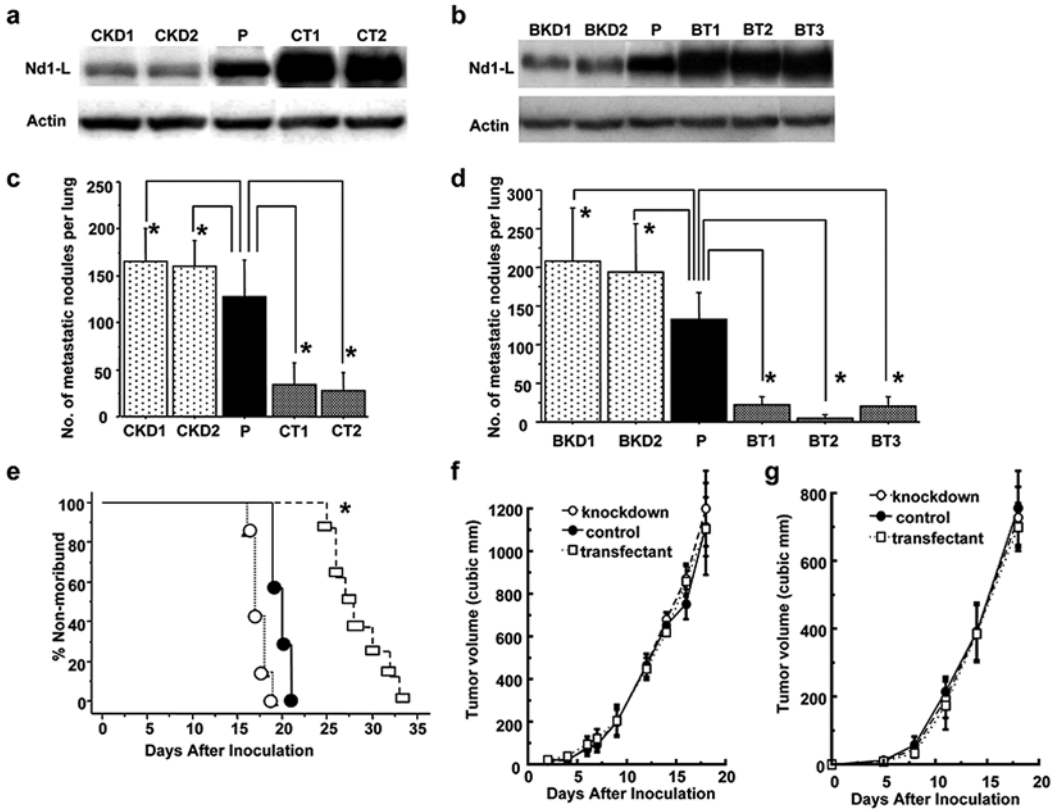

Figure 2. Nd1-L expression inversely correlates with the ability of cancer cells to metastasize to lung. (a and b) Nd1-L specific stable short hairpin (sh) RNAi expression vector (pBAsi-Nd1-L) or Nd1-L expression vector (pCR-2FLAG-Nd1-L) were introduced into Colon 26 cells (a) and B16 cells (b). Nd1-L protein in each transfectant was examined by Western blot analysis (CKD1 and CKD2, Colon 26 Nd1-L knock down clones; CT1 and CT2, Colon-26 Nd1-L transfectants; BKD1 and BKD2, B16 Nd1-L knock down clones; BT1, BT2 and BT3, B16 Nd1-L transfectants). P, control cells transfected with negative control vector. (c and d) Colon 26 (c) or B16 (d) cell population (a stable knockdown cell line, a control cell line and transfectant cell lines) were inoculated into lateral tail vein of BALB/c or C57BL/6 mice respectively. Two weeks after inoculation, mice were sacrificed and total number of lung metastatic nodules in individual mice was counted under the dissection scope. The vertical bar graph shows average number of metastatic nodules per lung for each group of mice (n=8-12). Data are shown ± SD. *P<0.05. (e) Survival curves of mice injected with Colon 26 transfected with pBAsi-Nd1-L vector (open circles; n=10), negative control vector (closed circles; n=10) and pCR-2FL-Nd1-L expression vector (open squares; n=11). Mice were monitored every day for signs of lung metastasis and respiratory failure. Mice with respiratory failure caused by lung metastasis, were considered moribund and euthanized. *P<0.05. (f and g) Growth curves of subcutaneously injected Colon 26 (f) and B16 (g) cells transfected with pBAsi-Nd1-L, pCR-2FL-Nd1-L, or negative control plasmids. Data are presented as the mean ± SD from three independent experiments.

and B16 cells (Fig. 2b; BKD1 and BKD2). We also obtained Nd1-L over-expressing Colon 26 cells (Fig. 2a; CT1 and CT2) and B16 cells (Fig. 2b; BT1, BT2 and BT3). These cells were injected into tail vein of 6-week-old syngeneic mice and number of metastatic nodules in lungs was examined 2 weeks after inoculation. Over-expression of Nd1-L dramatically reduced the number of lung metastatic nodules while knock down of Nd1-L promoted the metastasis in both Colon 26 and B16 cells (Fig. 2c and d respectively). The size of each nodule did not differ among Nd1-L over-expression, knock down, and control groups (data not shown). The number of moribund mice injected with Colon 26 Nd1-L transfectants was significantly lower than that of mice injected with control Colon 26 cells (Fig. 2e).

Although intravenous inoculation provides a convenient model in which to assay pulmonary metastases, its relevance to distant organ metastasis in animals and humans is limited. To determine the effect of Nd1-L expression on pulmonary metastasis in an orthotopic model, we inoculated B16 cells transfected either with pCR-2FL-Nd1-L vector (clone BT1 and BT2) or pCR-3.1 control vector into the back of syngeneic mice and tumor were removed after reaching >500 mm^3. As shown in Table I, over-expression of Nd1-L suppressed spontaneous pulumonary metastasis of B16 melanoma cells.

Table I. Lung metastasis after subcutaneous inoculation of B16 melanoma cells.

Cell line	Lung metastasis frequency	% metastasis
Control cells	5/7	71.4
Nd1-L transfectant 1 (BT1)	3/8	37.5
Nd1-L transfectant 2 (BT2)	2/8	25.0

In order to form orthotropic tumors, B16 cell population (a control cell line and two transfectant cell lines) were inoculated subcutaneously into the back of each mouse and tumors were removed after reaching >500 mm^3. Mice were sacrificed and lung metastasis was examined 3 weeks after removal of the primary tumor.

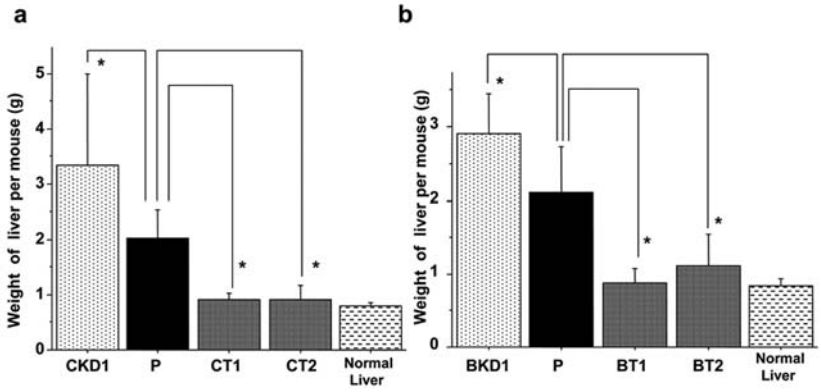

Figure 3. Nd1-L expression suppressed liver metastasis. (a) Nd1-L knock down (CKD1), parental (P), or Nd1-L over-expressed (CT1 and CT2) Colon 26 cells were inoculated into spleen of BALB/c mice. Ten days after inoculation, mice were sacrificed and the weight of liver in each mouse was measured. The vertical bar graph shows average weight of liver per mouse for each group of mice (n=6). (b) Nd1-L knock down (BKD1), parental (P), or Nd1-L over-expressed (BT1 and BT2) B16 cells were inoculated into spleen of C57BL/6 mice and analyzed experimental liver metastasis as described (a). The vertical bar graph shows average weight of liver per mouse for each group of mice (n=8). Data are presented as the mean ± SD. *P<0.05.

The over-expression or knock down of Nd1-L in Colon 26 or B16 cells did not alter proliferation rates of these cells *in vivo* (Fig. 2f and g). These data rule out the possibility that the effects of Nd1-L on tumor metastatic ability were attributable to the different proliferation rates. Thus, increased expression of Nd1-L significantly decreased pulmonary metastasis, in both experimental and spontaneous lung metastasis models.

To examine whether effect of Nd1-L on metastatic ability is specific for lung metastasis, we used intra-splenic injection method to analyze liver metastasis, which is another favorable metastatic lesion of colon cancer and melanoma. As shown in Fig. 3, over-expression of Nd1-L suppressed liver metastasis while knock down of Nd1-L promoted that in both Colon 26 (Fig. 3a) and B16 (Fig. 3b) cells. Thus, the effect of Nd1-L on metastatic ability is not specific for lung metastasis.

Nd1-L regulates motility and invasion in Colon 26 and B16 cells. Since migratory and invasive properties of cells are important factors that determine cancer cell metastatic ability, it is necessary to examine whether Nd1-L would contribute to the motility and invasiveness of these cells. Colon 26 cells and B16 cells stably over-expressing Nd1-L or transfected with Nd1-L siRNA were subjected either to migration assays or to Matrigel invasion assays (Fig. 4). As shown in the figures, over-expression of Nd1-L in both Colon 26 (Fig. 4a; CT1 and CT2) and B16 cells (Fig. 4b; BT1 and BT2) suppressed migration through a membrane. Invasion capacity through Matrigel was also decreased in these tumor cell lines transfected with Nd1-L (Fig. 4c and d). Furthermore, knock down of Nd1-L by siRNA increased the number of cells that invade through Matrigel in both Colon 26 (Fig. 4c) and B16 (Fig. 4d) cells. Thus, the amount of Nd1-L in these cancer cell lines inversely correlated with the migration and invasive capacities.

Nd1-L down-regulates growth factor-induced Rho family protein activation. The Rho family of small GTPases has recently been implicated in the invasive and metastatic capacities of various cancer cells. It was, therefore, of interest to explore the possibility that GTP binding proteins of the

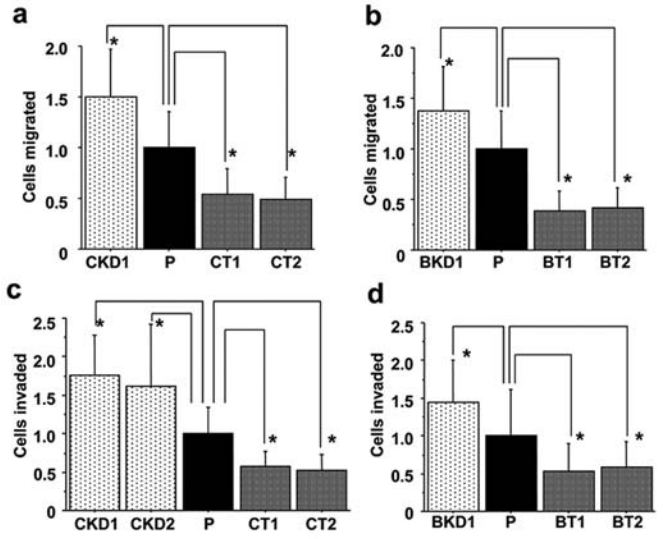

Figure 4. Nd1-L expression inhibits motility and invasion of cancer cells *in vitro*. (a and b) Relative *in vitro* migration activity of Colon 26 (a) and B16 (b) cells transfected with pBAsi-Nd1-L (CKD1, BKD1), control plasmid (P) or pCR-2FLAG-Nd1-L (CT1, CT2, BT1, BT2) toward serum. (c and d) Relative *in vitro* Matrigel invasion activity of Colon 26 cells (c) and B16 cells (d) expressing Nd1-L siRNA (CKD1, CKD2, BKD1), control mixed plasmid (P), or 2FL-Nd1-L (CT1, CT2, BT1, BT2). To compare the relative migration or invasiveness, values were normalized to that of control cell line. Data are presented as the mean± SD from three independent experiments. *P<0.05.

Rho family were involved in Nd1-L-mediated invasion or metastasis of cancer cells. As shown in Fig. 5, increased expression of Nd1-L inhibited the activity of RhoA, Rac1 and Cdc42 (relative amount of RhoA, Rac and Cdc42 in the active form), in NIH3T3 cells. The activities of these GTPases were not altered in cells transfected with the control vector.

To further explore the effect of Nd1-L on Rho activation by soluble factor, we measured RhoA activity in serum-starved NIH3T3 cells carrying either pBAsi-Nd1-L, pCR-2FL-Nd1-L or control vector after stimulation with lysophosphatidic acid (LPA), which is thought to activate Rho protein. Thirty seconds

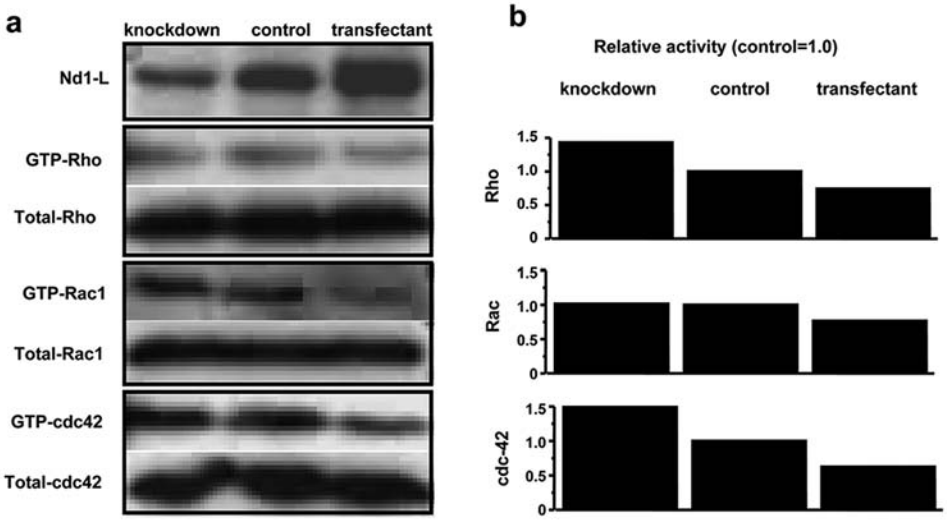

Figure 5. Nd1-L inhibits activities of Rho family proteins. (a) NIH3T3 cells were transfected with Nd1-L/siRNA, control plasmid or 2FL-Nd1-L, and 48 h later RhoA, Rac1, Cdc42 activity was measured. Western blots from one of the representative experiments showing activation of RhoA, Rac, and cdc42. (b) Relative activity of RhoA, Rac1 and cdc42 in each transfectant. Values represent RhoA, Rac1, Cdc42 activity relative to cells transfected with control plasmid. Data are presented as a representative of three independent experiments.

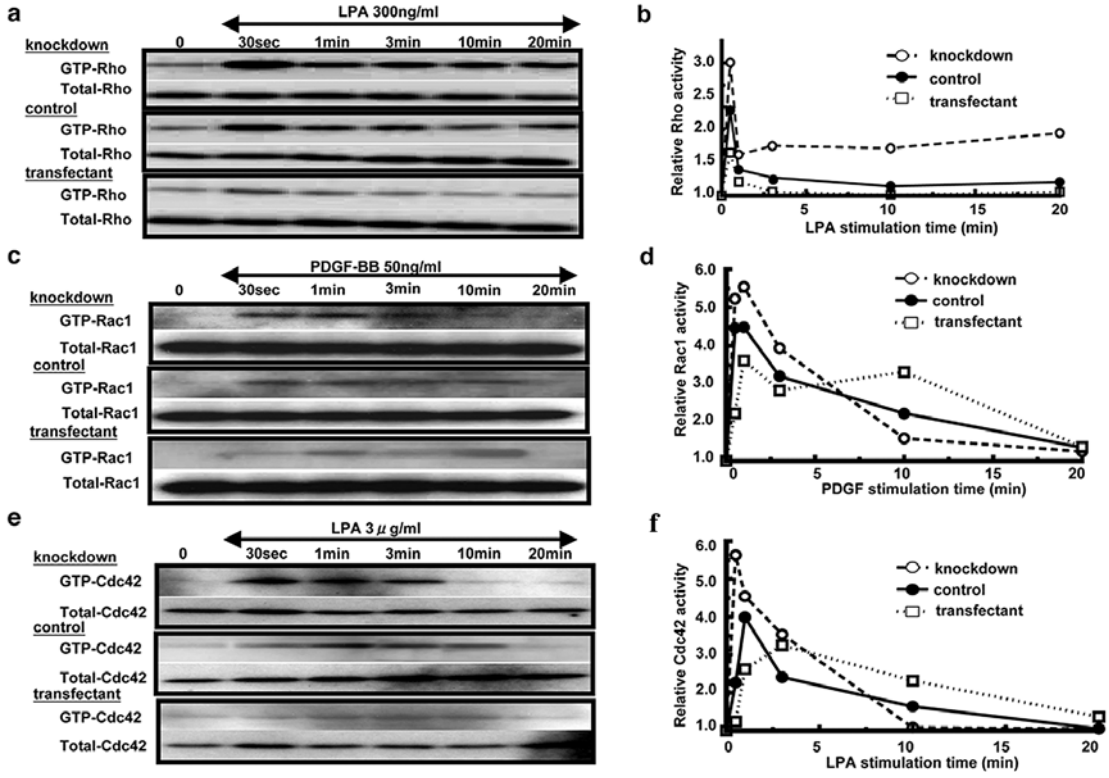

Figure 6. Nd1-L inhibits growth factor stimulated Rho family small GTPase activation. (a) NIH3T3 cells transfected with Nd1-L/siRNA (upper panel, knockdown), control plasmid (middle panel), or Nd1-L expression vector (lower panel) were stimulated with 300 ng/ml of LPA. RhoA activity was measured after stimulation in each time point. Western blots from one representative experiment are shown. (b) Relative RhoA activity was calculated by densitometry and plotted. (c) The same transfectants as (a) were stimulated with 50 ng/ml of PDGF-BB. Rac1 activity was measured after stimulation in each time point. Western blots from one representative experiment are shown. (d) Relative Rac1 activity was calculated by densitometry and plotted. (e) The same transfectants as (a) were stimulated with 3 μg/ml of LPA. Cdc42 activity was measured after stimulation at each time point. Western blots from one representative experiment are shown. (f) Relative cdc42 activity was calculated by densitometry and plotted. Data are presented as a representative of three independent experiments.

after the stimulation, Rho activity increased at maximum level and then declined. This increase of Rho activation was suppressed in NIH3T3 cells over-expressed with Nd1-L was initially of weaker magnitude (Fig. 6a and b). In contrast, initial Rho activation was augmented and high activity was sustained up to 20 min in NIH3T3 cells transfected with

pBAsi-Nd1-L. These data demonstrate that Rho activation by LPA stimulation was regulated in a Nd1-L-dependent manner and there is Nd1-L-dependent down-regulation of Rho following activation by LPA.

Rac and cdc42 induce reorganization of actin cytoskeleton at leading edge of the cell. We also measured Rac and cdc42 activity in serum starved NIH3T3 cells after stimulation with PDGF-BB and LPA respectively. Both Rac and cdc42 activation were augmented in pBAsi-Nd1-L transfected cells. In contrast, over-expression of Nd1-L suppressed Rac and cdc42 activation (Fig. 6c-f). Thus, Nd1-L negatively regulates Rho family GTPase activation, which may result in suppression of invasion and migration activity in cancer cells.

Discussion

The majority of cancer deaths result from tumor metastases rather than primary tumors. The biochemical mechanisms that regulate invasion and metastasis remain incompletely defined. In this report, we showed that over-expression of BTB-kelch protein Nd1-L suppressed cancer cell metastasis and prolonged survival of tumor bearing mice in an animal model. The metastatic process consists of multiple steps: i) dissociation of tumor cells from the primary site with a concomitant loss of cell to cell and cell to extracellular matrix (ECM) adhesions; ii) tumor cell adhesion to and subsequent local digestion of basement membrane; iii) retraction of endothelial cells and subsequent intravasation; iv) survival within the vasculature; v) extravasation from vasculature at a distant site, and vi) growth in a foreign or ectopic organ environment. 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 (27). Much attention has been paid to the interaction of cell to cell and cell to ECM adhesion on cancer metastatic capacity (28). However, over-expression of Nd1-L in cancer cells did not alter the expression of adhesion molecules such as integrin and cadherin family protein (data not shown). Tumor cell migration is an important step for establishment of cancer metastasis. Since over-expression of Nd1-L in Colon 26 and B16 cells reduced migration and invasion capacities *in vitro*, Nd1-L might control the intravasation and extravasation steps in the metastatic process. It is of interest that most of the lung metastatic nodules of intravenously injected Colon 26 cells expressed less Nd1-L protein than parental cells (Fig. 1). It is possible that reduction of Nd1-L protein is one of the critical factors for establishment of cancer metastasis.

Nd1-L might modulate activation of Rho family protein to suppress invasion and metastasis. Rho family GTPases, including RhoA, RhoC, Rac, and cdc42, are critical regulators of actin organization associated with cell motility, cell cycle progression, and gene expression (29). There is considerable evidence that aberrant Rho GTPase activation promotes tumor cell growth, invasion, and metastasis (30). We show here that Nd1-L suppresses the activation of the Rho family GTPase RhoA, Rac and cdc42, whereas, siRNA mediated knock-down of Nd1-L in NIH3T3 cells promote the activation of the Rho family GTPase. These observations strongly imply that Rho family GTPase acts downstream of Nd1-L. Although we have shown an important functional connection between Nd1-L and Rho family proteins, the nature of this connection is unclear. Nd1-L is recognized as an actin binding protein that belongs to the kelch family proteins. Pleiotrophic function of kelch family proteins other than actin binding has been demonstrated. Whether Nd1-L directly regulates Rho GEFs and how remains to be determined. Further biochemical characterization is required to elucidate the function of Nd1-L in regulation of Rho family protein and cytoskeletal organization.

Several genes responsible for metastasis have been identified. It is proposed that metastasis genes should be classified in two groups. First group of genes expressed in both primary and metastatic lesions. These genes confer both tumorigenicity of primary tumor and basal metastagenicity. The second set of genes act mainly as virulence genes that may allow tumors to aggressively invade, colonize and grow in the metastatic lesions without markedly contributing to primary tumor growth (22). Our result suggested that reduction of Nd1-L in tumor cells was one of the critical steps for the establishment of metastasis.

In conclusion, our results demonstrated that Nd1-L was able to affect tumor cell motility and invasion that contribute to the establishment of cancer metastasis via Rho family protein dependent mechanisms. The findings that Nd1-L over-expression can inhibit tumor cell migration and suppress metastasis provide clinical validation, potential prognostic tools and possible target for cancer treatment.

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