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DLC-1, a GTPase-activating protein for Rho, is associated with cell proliferation, morphology, and migration in human hepatocellular carcinoma

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Abstract

DLC-1 (deleted in liver cancer-1) is a tumor suppressor gene for hepatocellular carcinoma and other cancers. To characterize its functions, we constructed recombinant adenovirus encoding the wild-type DLC-1 and examined its effects on behaviors of a hepatocellular carcinoma cell line (SNU-368), which does not express DLC-1. Here, we found that restoration of DLC-1 expression in the SNU-368 cells caused an inhibition of cell proliferation with an increase of a subG1 population. Furthermore, DLC-1 overexpression induced disassembly of stress fibers and extensive membrane protrusions around cells on laminin-1. DLC-1 overexpression also inhibited cell migration and dephosphorylated focal adhesion proteins such as focal adhesion kinase (FAK), Cas (p130Cas; Crk-associated substrate), and paxillin. These observations suggest that DLC-1 plays important roles in signal transduction pathway regulating cell proliferation, cell morphology, and cell migration by affecting Rho family GTPases and focal adhesion proteins.

Keywords: DLC-1; Tumor suppressor gene; Cell migration; Cell morphology; GAP domain

The deleted in liver cancer (DLC-1) gene was identified from a primary human hepatocellular carcinoma (HCC) [1]. Since the first study of DLC-1 deletions in HCC, it has been reported that DLC-1 was down regulated by genomic deletions or DNA methylation in several types of cancer such as HCC, breast, colon, prostate, gastric, and non-small cell lung carcinoma [2–6]. In addition, several studies showed that the transfection of the DLC-1 gene caused significant growth inhibition and colony formation in various types of cancers [2,4,6,7] and abolished tumorigenicity in nude mice [6,7]. Therefore, it became clear that DLC-1 is a *bona fide* tumor suppressor gene.

Rho family GTPases play important roles in the regulation of a variety of cellular processes including cell cycle progression, gene expression, cytoskeletal organization, cell morphology, cell migration, and cell adhesion to extracellular matrix (ECM) [8]. Of the 24 known Rho family GTPases, RhoA, Rac1, and Cdc42 are representatively well-known for distinct functions [9]. Like other G proteins, Rho GTPases act as molecular switches that cycle between inactive GDP-bound and active GTP-bound states. This GDP/GTP cycle is regulated by at least three types of effector proteins including guanine nucleotide dissociation inhibitors (GDIs), guanine nucleotide exchange factors (GEFs), and GTPase-activating proteins (GAPs).

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GAPs were identified as molecules that enhance the intrinsic GTPase activity of Rho GTPases, thereby leading to their conversion to the inactive GDP-bound state [10].

DLC-1 cDNA sequence showed a high similarity to the rat p122RhoGAP sequence [11]. Transfection of p122RhoGAP, which has a GAP activity specific for RhoA, caused the disassembly of actin stress fiber, morphological rounding, and detachment [12], suggesting DLC-1 may be involved in the regulation of cytoskeletal rearrangement and morphological changes by inhibiting Rho GTPase activity.

Here, we found that DLC-1 have effects on cell proliferation, cell morphology, and cell migration. We also provide insight into the molecular mechanisms underlying these diverse functions of DLC-1.

Materials and methods

Cell culture. Human HCC cell lines and Human embryonic kidney cell line (HEK-293) were obtained from the Korean Cell Line Bank (Seoul, South Korea) and American Type Culture Collection (Rockville, MD, USA), respectively and maintained in RPMI-1640 supplemented with 10% fetal bovine serum and gentamicin (10 μg/ml) at 37 °C in a humidified 5% CO_2 atmosphere.

Northern blotting. For Northern blot analysis, mRNA was isolated as previously described [13]. Blots were hybridized with a random-labeled 1160-bp *Hin*dIII cDNA fragment of DLC-1 (GenBank Accession No. NM_006094) and a β-actin cDNA probe.

Construction of a recombinant adenoviral vector containing the DLC-1 gene. The DLC-1 cDNA (nt 430–3596) with a hemagglutinin (HA) epitope tag was amplified by PCR using pCMV/HA/DLC-1 plasmid as a template and transferred into the pShuttle vector of the Adeno-X Expression System (Clontech, Palo Alto, CA, USA). Recombinant DLC-1 and control LacZ adenoviruses were produced, purified, and tittered as previously described [14].

Cell proliferation assay. To examine the effect of DLC-1 on cell growth, SNU-368 cells were infected with either Ad/DLC-1 or Ad/LacZ at 20 MOI. The cells were trypsinized and counted with a Coulter Counter at the designed time.

Activity assays for RhoA GTPase. GTP-bound RhoA was determined by specific binding to GST-RBD fusion protein as previously described [15].

Immunofluorescence. SNU-368 cells cultured on coverslips were infected/transfected with the indicated gene and immunofluorescence assay was performed as previously described [16]. For study of DLC-1 induced morphological changes on ECMs, coverslips were pre-coated overnight at 4 °C with 20 μg/ml human fibronectin (Chemicon International, Temecula, CA), 20 μg/ml rat tail collagen-1 (Sigma), 20 μg/ml murine laminin-1 (Trevigen, Gaithersburg, MD, USA), or 0.01% (w/v) poly-L-lysine (Sigma) in PBS. SNU-368 cells infected with Ad/DLC-1 or Ad/LacZ for 12 h were trypsinized, incubated in suspension at 37 °C in RPMI containing 1% BSA with gentle rotation for 1 h, and transferred to the coverslips precoated with ECMs. After replating for 2 h, cells were fixed, stained with rhodamine-phalloidin, and visualized by a fluorescence microscope as described above.

Site-directed mutagenesis. The substitution of arginine (R718) to glutamate (E) in DLC-1 was obtained using the QuikChange site-directed mutagenesis system (Stratagene, La Jolla, CA, USA) with pCMV/HA/DLC-1 as template. The mutant cDNA sequences were confirmed by DNA sequencing.

Pharmacological screening. To identify intracellular signaling molecules modulating DLC-1 mediated morphological changes, cells were pretreated 30 min before the replating on laminin-1-coated coverslips with various pharmacological modulators, as followings (from Sigma): 10 μM cytochalasin D, 2 μM U0126, 10 μM LY294002, 10 μM SB203580, 30 μM cyclosporin A, 25 μM forskorin, 2 μM H89, 10 μM PP2, and 10 nM calyculin A. Cells were observed under a phase-contrast microscope 2 h after the replating.

Immunoblotting and immunoprecipitation. Western blot analysis was performed as previously described [16], using following antibodies: anti-FAK Ab, anti-phospho FAK (Y397 and Y925) Ab, anti-RhoA Ab, anti-Cdc42 Ab, and anti-β-tubulin Ab from Santa Cruz Biotechnology; anti-Rac1 Ab, anti-paxillin Ab, and anti-Cas Ab from BD Transduction Laboratories; anti-phospho paxillin (Y118) Ab from Cell Signaling Technology (Beverly, MA, USA); anti-phosphotyrosine Ab from Upstate Biotechnology (Placid, NY, USA); anti-HA antibody from Roche (Mannheim, Germany). For immunoprecipitation, the lysates were precleared with protein G–Sepharose (Amersham Biosciences) and precipitated with anti-Cas monoclonal antibody for 1 h at 4 °C followed by incubation with protein G–Sepharose for 1 h at 4 °C.

Cell migration assay. Monolayers of SNU368 cells were infected with recombinant adenovirus as described above. Twelve hours after the infection, a wound of ≈1 mm wide was made by scratching the monolayer with a pipette tip. Cell debris was removed by rinsing the cultures extensively with PBS and fresh medium. Wound closure was documented by photographing the cells under phase-contrast microscopy 30 h after the wounding. For migration assays using a transwell chamber (Corning Glassworks, Corning, NY, USA), the undersides of the membranes were coated with 10 μg/ml of each ECM proteins such as fibronectin, collagen-1, or laminin-1 for 1 h at 37 °C and then blocked with 1% BSA in RPMI for 1 h at 37 °C. The SNU-368 cells infected with Ad/LacZ or Ad/DLC-1 for 12 h were trypsinized, resuspended in RPMI with 1% BSA, and allowed to migrate to the undersides of the membranes for 2 h at 37 °C. Membranes were fixed in 3.7% paraformaldehyde for 10 min. Cells remaining on the upper sides of the membranes were removed using cotton swabs. The cells migrated were stained with Coomassie brilliant blue G (Sigma) and their images were taken.

Results and discussion

Inhibition of cell proliferation and increase of subG1 cells by DLC-1 overexpression in human HCC cell line

The expression of DLC-1 mRNA was examined in 11 human HCC cell lines by a Northern blot analysis. Nine of 11 cell lines showed 7.5 and 4.5 kb DLC-1 transcripts, while the other two cell lines (SNU-368 and -886) did not (Fig. 1A). To investigate the biological functions of DLC-1, we constructed a recombinant adenovirus expressing DLC-1 (Ad/DLC-1) and LacZ (Ad/LacZ), infected separately SNU-368 cells with either virus, and examined the effects of DLC-1 on cell proliferation. Overexpression of DLC-1 caused a significant inhibition of cell growth, compared with control cells infected with Ad/LacZ (Fig. 1B). Analysis of cell death by flow cytometry at 4 days after infection showed that 19% of the cells infected with Ad/DLC-1 had DNA contents less than 2n (i.e., subG1, an indicative of apoptosis), compared with 4% of the cells infected with Ad/LacZ (Fig. 1C). These results are consistent with a recent study reporting that DLC-1 expression induced apoptosis associated with the cleavage of caspase-3 and reduction of Bcl-2 expression [7]. Therefore, it can be suggested that DLC-1 functions as a tumor suppressor.

The effect of DLC-1 on the change of cell morphology

Rho family GTPases are well known to play key roles in cell shape change through their effects on the actin cyto-

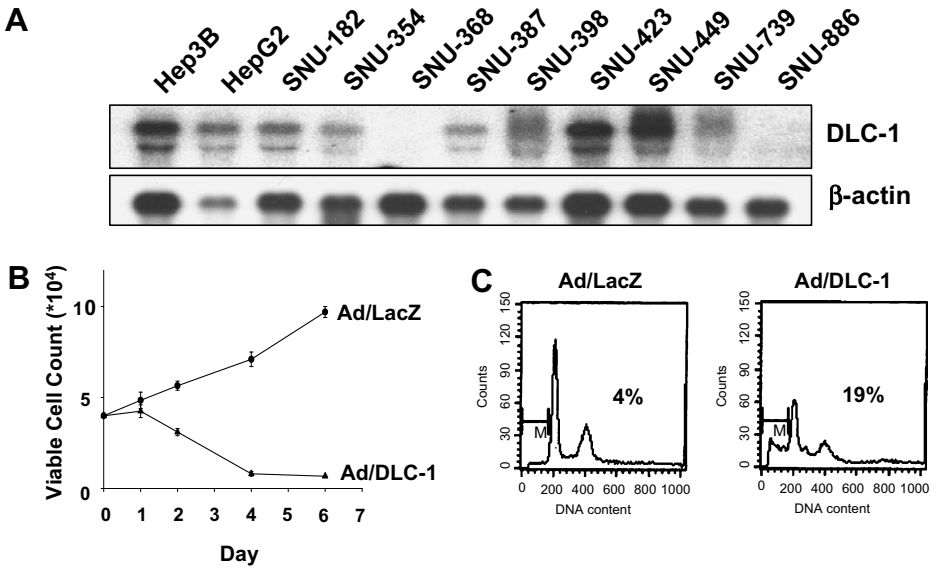

Fig. 1. DLC-1 inhibits cell proliferation in SNU-368 human HCC cell line. (A) The expression of DLC-1 mRNA was determined in 11 human hepatocellular carcinoma cell lines by Northern blot analysis. β-Actin was analyzed for quantifying the RNA loaded in each lane. (B) SNU-368 cells were infected with Ad/DLC-1 or Ad/LacZ at a MOI of 20 and cell numbers were determined using a Coulter Counter at the indicated days. (C) SUN368 cells infected with Ad/DLC-1 or Ad/LacZ were subjected to flow cytometry at 4 days after infection and cell death was observed by measuring the sub G1 proportion of cells.

skeleton. Overexpression of p122RhoGAP resulted in the cell morphological changes and the disassembly of actin stress fiber by inhibiting RhoA activity [12]. We thus expected DLC-1 to cause cell morphological changes. As we expected, the overexpression of DLC-1 induced cell rounding and branched membrane protrusions, whereas Ad/Lacz infection did not cause any morphological changes in SNU-368 cells (Fig. 2A). In addition, stress fibers were not formed in cells infected with Ad/DLC-1, but formed in the cells infected with Ad/LacZ. In our attempt to examine the effects of DLC-1 on RhoA activity, we found that the expression of DLC-1 clearly reduced the amounts of GTP-RhoA compared to those of Ad/LacZ-infected cells (Fig. 2B). Most RhoGAP-containing proteins have a con-

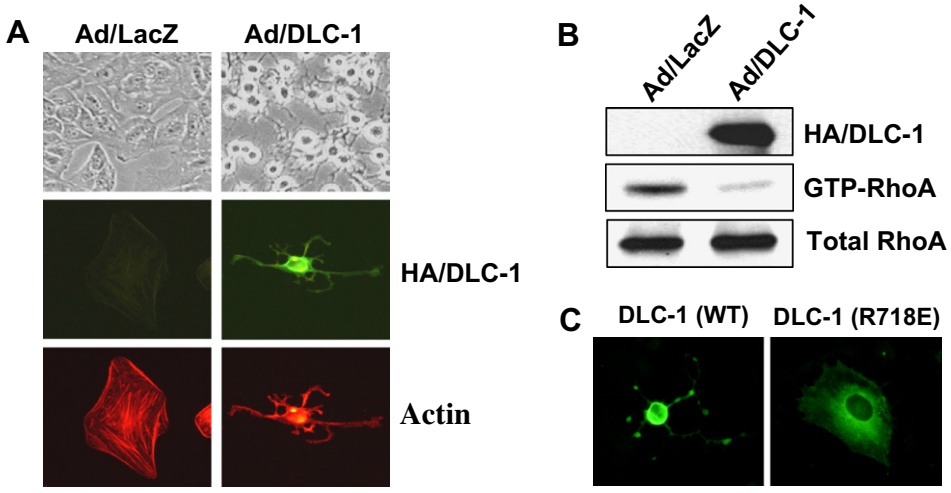

Fig. 2. Morphological alterations induced by DLC-1 overexpression. (A) SNU-368 cells infected with Ad/DLC-1 or Ad/LacZ virus for 12 h were observed by a light microscope (top panel), or stained with anti-HA antibody followed by FITC-conjugated rabbit anti-mouse IgG antibody and rhodamine-phalloidin to visualize DLC-1 and actin, respectively. (B) The amounts of active GTP-bound Rho GTPases were determined by the GST pull-down assay employing GST-RBD to precipitate GTP-bound RhoA from the lysates of SNU-368 cells infected with Ad/DLC-1 or Ad/LacZ. An aliquot of each lysate was checked for the DLC-1 expression with anti-HA antibody (top panel). (C) The arginine at position 718 of the wild-type DLC-1 was mutated to glutamate (R718E) by site-directed mutagenesis. SNU-368 cells were transfected with pCMV/HA/DLC-1 (WT) or pCMV/HA/DLC-1 (R718E) plasmids and stained with anti-HA antibody followed by FITC-conjugated rabbit anti-mouse IgG antibody. Cells were examined under a fluorescence microscope.

served arginine residue, termed the "arginine finger" in the active site of RhoGAP domain, stabilizing the transition state of GTP hydrolysis [17]. Mutagenesis study of p122RhoGAP identified that the residues Arg-668, Lys-706, or Arg-710 are important for the morphological changes induced by p122RhoGAP [12]. To examine whether the RhoGAP domain of DLC-1 is responsible for the cell morphological changes, we constructed a DLC-1 mutant in which a putative active arginine residue in the GAP domain (R718, corresponding to R710 of rat122RhoGAP) is replaced by glutamic acid. As shown in Fig. 2C, transient expression of the mutant DLC-1 (R718E), unlike wild-type DLC-1, did not cause the morphological changes, indicating that the RhoGAP domain of DLC-1 is important for regulating cell morphology. These data suggest that DLC-1 regulate cell morphology and actin cytoskeletal organization through down-regulating RhoGTPases.

Promotion of membrane protrusion mediated by DLC-1 expression on laminin-1-coated coverslips

Maintenance of cellular morphology via actin cytoskeletal rearrangement is well known to be regulated by signal transduction via integrin engagement to ECM proteins [18]. We next investigated the effects of ECM proteins on the morphology and actin cytoskeleton of SNU-368 cells infected with Ad/DLC-1 or Ad/LacZ. The cells infected separately with each virus were trypsinized and replated on a poly-lysine, fibronectin, collagen-1, or laminin-1. Two hours after the replating, cells were stained with rhodamine-conjugated phalloidin and observed by fluorescence microscope. LacZ-infected cells replated on the ECM proteins were well spread and had prominent stress fibers. In contrast, DLC-1 infected cells showed branched membrane protrusions on a surface coated with fibronectin or collagen-1 (data not shown). These membrane structures were especially prominent in cells replated on a surface coated with laminin-1 (Fig. 3A). These results suggest that the membrane protrusions induced by DLC-1 are more efficient in signal transduction via specific integrin subtypes, especially laminin-1-binding integrins.

To examine the signaling molecules regulating DLC-1-induced membrane protrusions in response to laminin-1, we pretreated cells with pharmacological modulators of intracellular signaling molecules before their replating and examined whether these modulators could abolish the DLC-1 induced membrane protrusions on laminin-1 (see Materials and methods). As shown in Fig. 3B, the membrane protrusions are blocked by treating cells with an actin cytoskeletal inhibitor cytochalasin D, indicating that intact cytoskeletal organization is importantly involved in the DLC-1 mediated morphological changes. Furthermore, a Src family kinase inhibitor (PP2) or a serine/threonine phosphatase inhibitor (calyculin A) also blocked the DLC-1-mediated morphological changes, while having no significant effects in the cells treated with the other inhibitors we treated (data not shown), suggesting that Src kinase and serine/threonine phosphatase activity are critical for the DLC-1-mediated morphological changes on laminin-1. These results are consistent with a previous study demonstrating that p190RhoGAP mediates a Src-dependent signal for neurite outgrowth in neuroblastoma which is promoted by the ECM protein laminin-1 [19]. It is also known that tyrosine phosphorylation of p190RhoGAP by Src kinase initiates its association with p120RasGAP and activation of its catalytic GAP activity [20]. Therefore, we propose that Src activation and subsequent tyrosine phosphorylation of DLC-1 is necessary for integrin-mediated membrane protrusions.

DLC-1 regulates cell migration and led to the dephosphorylation of focal adhesion proteins

Rho family GTPases are well known to regulate cell migration. Recently, Goodison et al. [21] found that restoration of the DLC-1 in metastatic sublines of MDA-MB-435 breast cancer cells resulted in the inhibition of

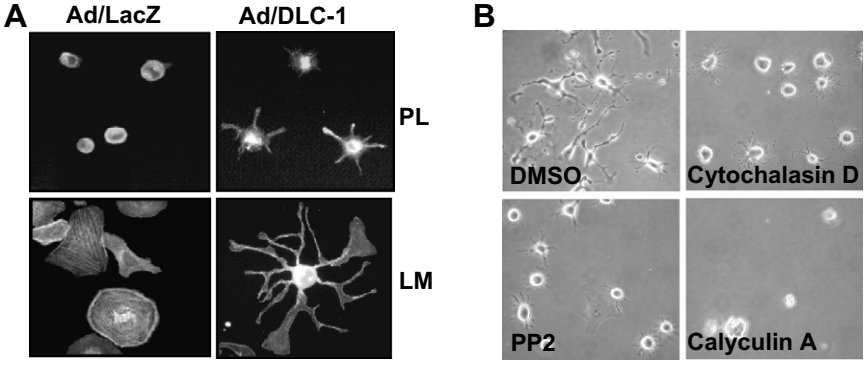

Fig. 3. Membrane protrusions mediated by DLC-1 on laminin-1-coated plates are blocked by PP2 or calyculin A. (A) SNU-368 cells infected with Ad/LacZ or Ad/DLC-1 were trypsinized and replated onto poly-L-lysine (PL) or laminin-1 (LM) -coated coverslips. Two hours after replating, cells were fixed, stained with rhodamine-phalloidin, and visualized by fluorescence microscope. (B) Ad/DLC-1 infected cells were pretreated for 30 min before replating on laminin-1-coated coverslips with pharmacological modulators as follows: 10 μM cytochalasin D, 10 μM PP2, and 10 nM calyculin A.

migration and invasion in vitro and a significant reduction in the ability of forming pulmonary metastases in athymic mice. In addition, Wong et al. [22] reported that DLC-1 expression reduced the migration and invasiveness of SMMC-7721 HCC cells. To test whether DLC-1 is involved in the migration of SNU-368 cells, the cells were infected with Ad/DLC-1 or Ad/LacZ for 12 h, a wound through the cell monolayer was introduced using a pipette tip, and wound healing was observed for 30 h. When infected with Ad/DLC-1, SNU-368 cells showed significant inhibition of migration to the center of the wound. In contrast, cells infected with Ad/LacZ were able to migrate to the center of the wound (Fig. 4A). In addition, the ability of cells to migrate toward fibronectin, collagen-1, or laminin-1 was examined using transwell migration assays. Haptotactic migration of SNU-368 cells toward the ECM proteins was inhibited by DLC-1 overexpression, compared to the cells infected with Ad/LacZ (Fig. 4B). Together, the data indicate that DLC-1 inhibits the migration of SNU-368 cells.

To define molecular basis for the impaired migration of cells by DLC-1, we observed the effects of DLC-1 on the proteins localized at focal adhesions which are formed by interaction of integrins with ECM and actin cytoskeletal proteins and play regulatory roles in cell migration [23]. Especially, very recent studies reported that DLC-1 localizes to focal adhesions [24,25]. FAK is known to be important in regulating focal adhesion dynamics during cell migration [26]. In particular, tyrosine phosphorylation of FAK at Y397 and the subsequent tyrosine phosphorylation of other focal adhesion complex-associated proteins such as paxillin and Cas are required for focal adhesion formation and cell migration [27–29]. As shown in Fig. 4C, the expression of DLC-1 led to the dephosphorylation of tyrosine residues of FAK (Y397), paxillin (Y118), and Cas in the lysates of SNU-368 cells infected with Ad/DLC-1 but did not affect their expression levels. In this regard, it is likely that the dephosphorylation of focal adhesion proteins by DLC-1 could lead to a decrease in overall focal adhesion turnover, resulting in a decrease in the cell migration. We also observed that DLC-1 induced the dephosphorylation of FAK (Y925), which generates a docking site for Grb2 [30]. The interaction with Grb2 leads to the recruitment of SOS and activation of the Ras-MAPK sig-

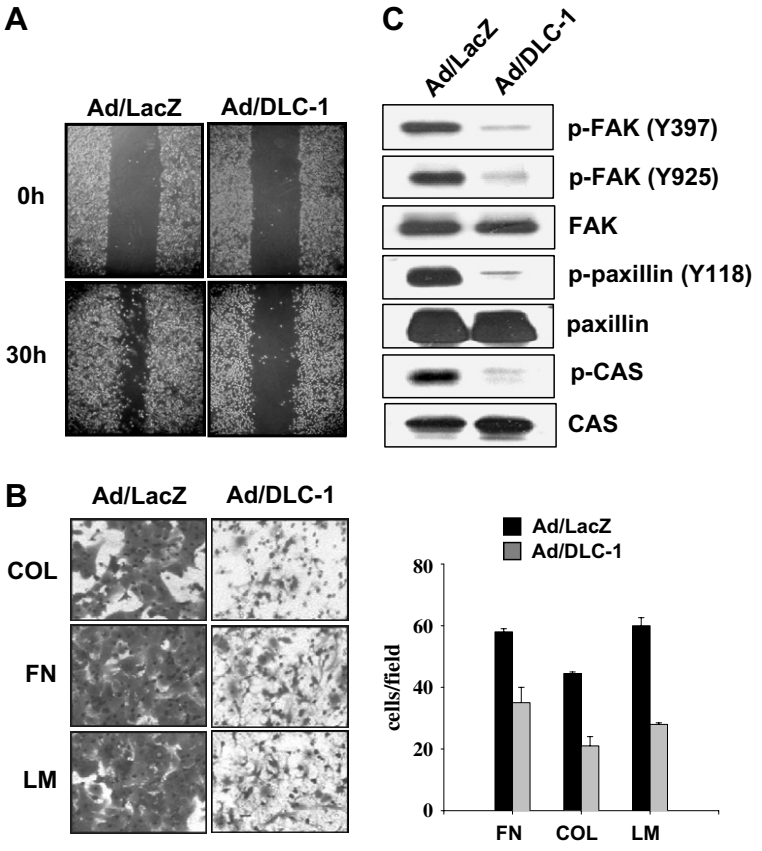

Fig. 4. DLC-1 inhibits cell migration and stimulates dephosphorylation of proteins localized in focal adhesion. (A) SNU-368 cells were infected with Ad/DLC-1 or Ad/LacZ for 12 h and a wound was generated as described under Materials and methods. Wound healing was observed for 0 and 30 h after the wound generation. (B) Haptotactic cell migration toward fibronectin (FN), collagen-1 (COL), or laminin-1 (LM) was assayed for 2 h in the transwell chambers. The number of migrated cells was counted under the microscope. Hapotactic migration assay were performed two times in triplicate. (C) Cell lysates from SNU-368 cells infected with Ad/DLC-1 or Ad/LacZ for 12 h were analyzed by immunoblotting with the antibodies indicated on the right each panel. The anti-Cas immunoprecipitates from lysates prepared from Ad/DLC-1 or Ad/LacZ infected SNU-368 cells were immunoblotted with anti-phophotyrosine or Cas antibodies.

nal transduction pathway [31], thus it will be of interest to examine the effects of DLC-1 on the Ras-MAPK pathway.

In summary, we demonstrated that DLC-1 restoration in DLC-1 negative SNU-368 human HCC cells resulted in inhibition of cell proliferation and migration, and induction of cell morphological changes. The effects involved the suppression of Rho GTPase activity and dephosphorylation of focal adhesion proteins. Further studies need to be focused on understanding the regulation of DLC-1, as well as the detailed molecular mechanism(s) by which DLC-1 affects cell proliferation and migration.

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