

The role of integrin-linked kinase in melanoma cell migration, invasion, and tumor growth

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Abstract

Melanoma is a life-threatening disease with a high mortality rate due to rapid metastasis. Currently, there is no effective treatment for metastatic melanoma. Integrin-linked kinase (ILK) is a serine/threonine kinase and has its role implicated in connecting cell-extracellular matrix interaction and growth factor signaling to cell survival, cell migration, invasion, anchorage-independent growth, angiogenesis, and epithelial-mesenchymal transition. However, the functional role of ILK in melanoma progression is not completely understood. We have previously shown that strong ILK expression was significantly associated with melanoma thickness. In this study, we further elucidate the role of ILK in melanoma cell migration, invasion, anchorage-independent growth, and tumor growth *in vivo* by specific ILK knockdown using small interfering RNA and short hairpin RNA. We found that ILK knockdown impeded melanoma cell migration, which was associated with reduced stress fiber formation, cell spreading, and cell adhesion. Furthermore, ILK knockdown decreased the invasion ability of melanoma cells and the formation of anchorage-independent colonies in soft agar. Moreover, ILK knockdown significantly impaired the growth of melanoma xenografts in severe combined immunodeficient mice. This study highlights the importance of ILK in melanoma progression and provides an attractive target for the treatment of melanoma. [Mol Cancer Ther 2007;6(6):1692–700]

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Introduction

Melanoma is an invasive skin cancer and is potentially life-threatening. Although it accounts for 4% of all dermatologic cancers, it is responsible for 80% of deaths from skin cancer. The major mortality is due to tumor metastasis; only 14% of patients with metastatic melanoma survive for 5 years (1). From tissue microarrays, we discovered that integrin-linked kinase (ILK) expression increases with melanoma thickness and lymph node invasion (2). ILK is a serine/threonine kinase that interacts through its COOH terminus with $\beta 1$ and $\beta 3$ integrins, which are receptors for extracellular matrix (ECM) proteins like collagen and laminin (3). ILK, via its COOH terminus, also interacts with actin cytoskeleton proteins through association with CH-ILKBP-actopaxin, paxillin, and affixin, which are responsible for focal adhesion formation (4–8). In addition, ILK interacts with growth factor receptors through the association of its four ankyrin repeats at the NH₂ terminus with PINCH and Nck-2 adaptor proteins (9). ILK also contains a pleckstrin homology-like domain which binds phosphatidylinositol triphosphate (3–5) and regulates its kinase activity. Furthermore, ILK binds to PKB/Akt and GSK-3 and controls their activities through phosphorylation (10). ILK phosphorylates and activates Akt at Ser⁴⁷³, which regulates genes essential for survival (11–13). ILK also phosphorylates GSK-3 at Ser⁹ and inactivates it, leading to the activation of activator protein transcription factor, cAMP-responsive element binding protein, or β -catenin/TCF transcription factor, and the expression of cyclin D1 and matrix metalloproteinase-9 (MMP-9; refs. 14–16). In addition, ILK overexpression has been shown to promote anchorage-independent survival (17). Therefore, ILK plays an important role in linking extracellular signaling to the regulation of survival, cell cycle progression, migration, and invasion.

We have previously shown that ILK expression is significantly increased in melanoma compared with benign nevi (2). To further investigate the role of ILK in melanoma progression and determine if ILK can be used as a therapeutic target, we specifically knocked down ILK expression by small interfering RNA (siRNA) in metastatic melanoma MMRU cells and also generated short hairpin RNA (shRNA) stable clones from this cell line. We found that ILK knockdown impeded melanoma migration, reduced stress fiber formation, and changed cell morphology. ILK knockdown also decreased the cell attachment ability of melanoma cells to fibronectin, inhibited invasion through Matrigel and MMP-9 activity, and suppressed anchorage-independent growth in soft agar. Finally, we showed that ILK knockdown could inhibit tumor growth *in vivo* by 80% to 90%. These data suggest that ILK plays a key role in melanoma progression and may serve as a potential molecular target in melanoma treatment.

Materials and Methods

Cell Culture and Antibodies

Melanoma cell line MMRU and Sk-mel-110 were gifts from Dr. H.R. Byers at Boston University (Boston, MA) and Dr. A.P. Albino at Memorial Sloan-Kettering Cancer Center (New York, NY), respectively. Cells were cultured in DMEM (Invitrogen) supplemented with 10% fetal bovine serum (Hyclone), 100 units/mL of penicillin, and 100 µg/mL of streptomycin in 5% CO₂ humidified atmosphere at 37°C. ILK antibody was purchased from Cell Signaling.

siRNA

A 21-bp double-stranded siRNA molecule specifically targeting the integrin-binding domain of ILK or a control nonsilencing sequence was synthesized (Qiagen; ref. 13). Cells at 40% confluency were transfected overnight with 25 nmol/L of siRNA using SiLentFect (Bio-Rad).

shRNA Construct Cloning and Stable Transfection

The shRNA sequences of ILK corresponded to positions 472 to 492 of the human ILK gene (National Center for Biotechnology Information accession number NM_004517). The sense oligonucleotide containing the ILK targeting sequence (underlined) was 5'-GATCCCTCTCAACCG-TATTCCATACTTCAAGAGAGATGGAATACGGTT-GAGATTTTA-3', and the antisense oligonucleotide is: 5'-AGCTTAAAAATCTCAACCGTATCCATACTCTCTT-GAAGTATGGAATACGGTTGAGAGGG. To generate a shRNA duplex, the sense and antisense oligonucleotides were annealed and double-stranded oligonucleotides were cloned into the pSUPERIOR-neo plasmid (OligoEngine). To establish ILK shRNA-stable clones, the pSUPERIOR-ILKshRNA plasmid or the vector alone was transfected into MMRU cells using Effectene (Qiagen). Cells were selected with medium containing 800 µg/mL of G418 by limiting dilution method to ensure that the clones were from a single cell. All clones were maintained in medium supplemented with 400 µg/mL of G418.

Cell Migration Assay

Cell migration was determined by wound healing assay. After cells reached confluency, a wound was made in the monolayer by pressing a razor blade down on the plate to mark it; the blade was used to remove the cell monolayer from one side. The debris was removed by washing the monolayer twice with serum-free medium and the cells were cultured for an additional 24 to 36 h. Cell migration was recorded using a microscope. The number of cells which migrated across the wound mark was counted in five different microscopic fields.

Immunofluorescence

ILK shRNA-stable clone or vector control cells were seeded on coverslips and serum starved overnight. Cells were stimulated with 10% fetal bovine serum for 60 min, then fixed and permeabilized with 2% paraformaldehyde and 0.5% Triton X-100 in PBS for 10 min. Cells were stained with 1 unit of phalloidin-rhodamine (Invitrogen) for 30 min and Hoechst 33258 (20 mmol/L) for 5 min. Fluorescence staining was visualized with Zeiss fluorescent microscope and photos taken with a 12-bit Retiga Ex camera using

Northern Eclipse imaging software. Stress fibers were quantified by ImageJ software (NIH) from photos taken randomly in 10 fields. Five cells were quantified in each field.

Rho GTPase Activation Assay

The GST-PAK1 p21-binding domain (GST-PBD) and GST-Rhotekin Rho-binding domain (RBD) were prepared as described previously (18, 19). Activation of Rac, Rho, and Cdc42 were analyzed by pulldown assays. MMRU cells were washed twice with ice-cold PBS and lysed for 5 min in 350 µL of lysis buffer [50 mmol/L Tris (pH 7.5), 150 mmol/L NaCl, 1% Triton X-100, 10 mmol/L MgCl₂, 10% glycerol, EDTA-free protease inhibitor cocktail (Roche Diagnostics), 5 mmol/L sodium fluoride, and 1 mmol/L sodium orthovanadate]. The samples were clarified by centrifugation at 12,000 × g at 4°C for 15 min and equivalent amounts of each cell lysate adjusted to 500 µL were incubated with 20 µg of GST-RBD beads for capturing active Rho or GST-PBD beads for capturing active Rac and Cdc42 at 4°C for 45 min to 1 h. The beads were collected and washed thrice with lysis buffer and subsequently eluted with SDS sample buffer. Active Rac, Rho, and Cdc42 were detected by immunoblot with an anti-Rac1 antibody (Upstate), anti-RhoA antibody (Upstate), and anti-Cdc42 antibody (Upstate).

Cell Attachment Assay

Ninety-six-well plates were coated with 1.25 µg/mL of fibronectin (Sigma) in 100 µL PBS overnight at 4°C. Wells coated with bovine serum albumin served as negative controls. The plates were blocked with 2.5 mg/mL of bovine serum albumin for 2 h in DMEM at 37°C. Cells were trypsinized and 2 × 10⁴ cells were seeded in each well for 1 h at 37°C. Unattached cells were discarded and the attached cells were gently washed thrice with PBS. Cells were fixed with 1% formaldehyde for 10 min and stained with 0.5% crystal violet for 30 min and solubilized with methanol. Relative number of attached cells was measured by ELISA plate reader at 590 nm.

Cell Invasion Assay

Polycarbonate membranes (8.0 µm pore size) of the upper compartment of 24-well Transwell culture chambers were coated with 18 µL of 5 mg/mL Matrigel (BD Biosciences) in serum-free medium. Cells (5 × 10⁴) suspended in 250 µL of serum-free medium were applied on the upper compartment, and the lower compartment was filled with 750 µL of DMEM containing 10% fetal bovine serum. After 24 h of incubation, cells were fixed with 10% trichloroacetic acid at 4°C for 1 h. Noninvaded cells on the upper surface of the filter were removed carefully with a cotton swab. Invading cells on the lower side of the filter were stained with 0.5% crystal violet for 2 h and the stained filters were photographed. The crystal violet dye retained on the filters was extracted with 30% acetic acid and cell invasion was measured by reading the absorbance at 590 nm.

Soft Agar Assay

Culture plates (35 mm) were first layered with 0.5% noble agar (Sigma) diluted in DMEM containing 10% fetal bovine serum. Cells (1.3 × 10⁴) were suspended in 1 mL of 0.3%

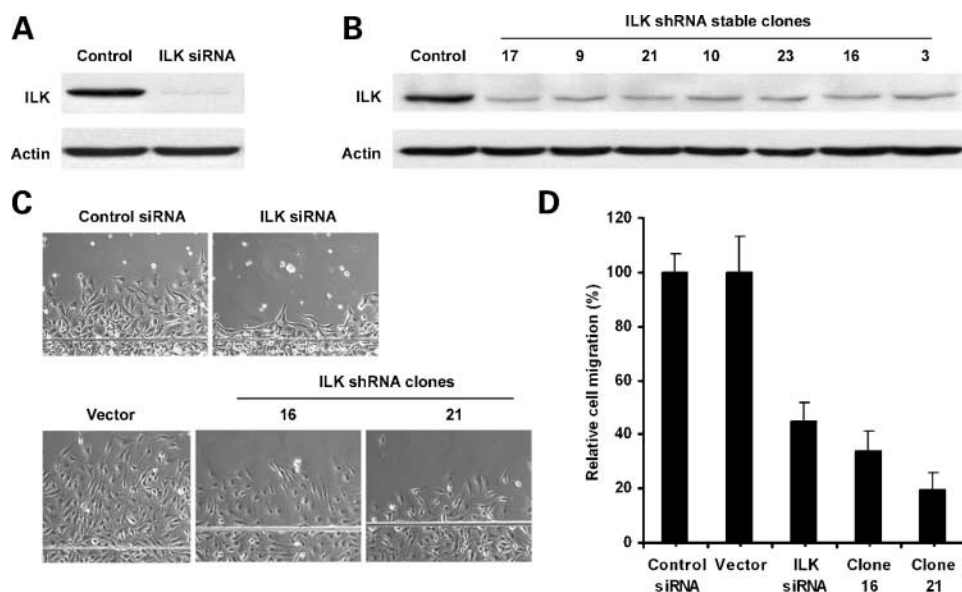


Figure 1. Silencing of ILK expression by ILK siRNA and ILK shRNA inhibited MMRU cell migration. **A**, inhibition of ILK expression by siRNA. MMRU melanoma cells were transfected with 25 $\mu\text{mol/L}$ of nonsilencing control siRNA or siRNA targeting the integrin-binding domain of ILK. Ninety-six hours after transfection, protein expression was analyzed by Western blotting. **B**, inhibition of ILK expression in stable clones by Western blotting. **C**, cell migration assay on siRNA-treated MMRU cells (*top*) and ILK shRNA-stable clones (*bottom*). A wound was created in the confluent cell monolayer by using a razor blade. After 24 h, cells were photographed (magnification, $\times 100$). **D**, quantification of cell migration. Cells which migrated across the wound were scored in five different microscopic fields. *Columns*, mean; *bars*, SD.

agar in the same medium and was poured on the bottom agar. Plates were maintained in a humidified incubator for 15 days and were photographed with the inverted Zeiss Axiovert 200 microscope. Colonies with more than 30 cells or cells larger than 50 μm were counted from three random square grids. Experiments were done in triplicate.

Zymography

Cells (2.5×10^6) were seeded in 100 mm plates for 24 h. The proteins in the conditioned medium were concentrated with YM-30 centricon membranes (Millipore) at $5,000 \times g$ for 4 h at 4°C . Proteins (25 μg) were loaded in nonreducing conditions on a 10% polyacrylamide gel containing 0.1% gelatin (Sigma). After electrophoresis, gels were incubated in Triton X-100 exchange buffer [20 mmol/L Tris-HCl (pH 8.0), 150 mmol/L NaCl, 5 mmol/L CaCl₂, and 2.5% Triton X-100] for 30 min followed by a 10 min wash thrice with the incubation buffer (same buffer without Triton X-100). Gels were incubated in incubation buffer overnight at 37°C , stained with 0.5% Coomassie blue R250 (Sigma) for 1 h and destained with 30% methanol and 10% glacial acetic acid for 1 h. Gelatinolytic activity was shown as clear areas in the gel. Recombinant MMP was used as a positive control.

Tumor Xenograft *In vivo*

Tumors were induced by injecting 4×10^6 melanoma cells s.c. into the flanks of 12-week-old male severe combined immunodeficient mice. The tumor growth was measured by a caliper when palpable. Tumor volume was calculated according to the formula: $V = L \times W^2 \times \pi/6$ (20). Mice were sacrificed 60 days after injection.

Results

ILK Knockdown Impedes Melanoma Cell Migration

To study the role of ILK in melanoma cell migration, we specifically knocked down ILK expression using ILK

siRNA which targets the integrin-binding domain of ILK (13). In Fig. 1A, ILK siRNA was able to knock down ILK protein expression by >70% in MMRU cells. ILK knockdown by siRNA impeded the ability of MMRU cell migration by 50% (Fig. 1C and D). To further confirm this result, we generated ILK shRNA-stable clones. We, for the first time, discovered that the 21-mer nucleotide sequence targeting the ILK transcript at the 472 to 492 position was able to successfully knock down ILK protein expression (Fig. 1B). Similar to ILK siRNA treatment, ILK knockdown stable clones 16 and 21 showed significant reduction in cell migration by 60% and 80%, respectively (Fig. 1C and D). To confirm that the reduced number of cells in ILK shRNA-stable clones across the wounded area was due to the inhibition of cell migration, not the inhibition of cell proliferation by ILK shRNA, we stained the cells with the human nuclear antigen Ki-67. Our data indicate that the cell proliferation rates were similar between control cells and the ILK shRNA-stable clones (Fig. 2A). We also confirmed this by cell proliferation assays (Fig. 2B). To further verify the role of ILK in melanoma cell migration, we treated the MMRU and Sk-mel-110 melanoma cells with a pharmacologic inhibitor, KP392, which inhibits ILK kinase activity. Consistent with the RNAi experiments, KP392 decreased melanoma cell migration in both MMRU and Sk-mel-110 cells (Supplementary Fig. S1).³ These data indicate that ILK is important in melanoma cell migration.

ILK Regulates Stress Fiber Formation and Cell Morphology through Rho GTPase Family Members, Rac and Cdc42

It has been shown that inhibition of ILK kinase activity by ILK inhibitor impeded cell attachment and filamentous

³ Supplementary material for this article is available at Molecular Cancer Therapeutics Online (<http://mct.aacrjournals.org/>).

actin organization (21). We confirmed such a finding in melanoma cells (Fig. 3A). ILK knockdown reduced stress fiber formation by 20% after serum stimulation (Fig. 3B). More drastically, ILK knockdown of stable clone 21 cells displayed different cell morphologies and stress fiber organizations when compared with the control cells. There were more stress fibers running across the cells in the control although the fibers in ILK knockdown cells mainly localized at the periphery of the cells. Control cells also showed a higher degree of cell spreading when compared with the ILK knockdown cells (Fig. 3A). There were also less lamellipodia and filopodia formation in ILK knockdown cells. It has recently been shown that cell spreading

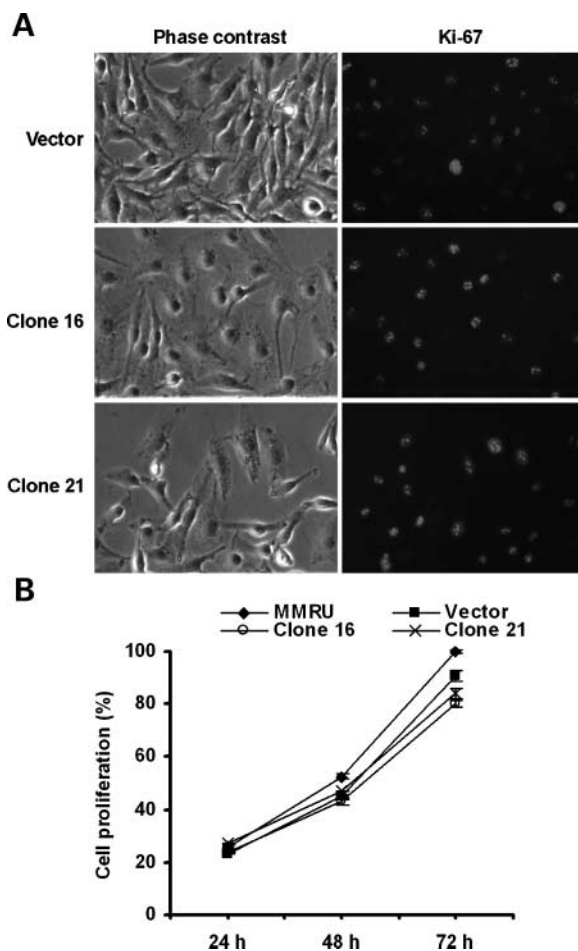


Figure 2. ILK shRNA did not alter cell proliferation. **A**, immunofluorescence images of Ki-67 staining. Cells were fixed, permeabilized, and blocked with blocking reagent for 30 min at room temperature followed by incubation with a monoclonal mouse anti-Ki-67 primary antibody and with Cy5-conjugated goat anti-mouse IgG. Microscopy was done on a Zeiss Axiovert 200 inverted microscope. **B**, cell proliferation of ILK shRNA-stable clones. Parental MMRU cells, vector control stable cells, and ILK shRNA-stable clones were grown in 24-well plates for 24, 48, and 72 h. Cells were then fixed with trichloroacetic acid and were stained with sulforhodamine B. Cells were washed with 1% acetic acid and air-dried. The dye was solubilized and cell proliferation was determined by spectrophotometric readings at 550 nm. Points, means from sextuplet samples; bars, SD.

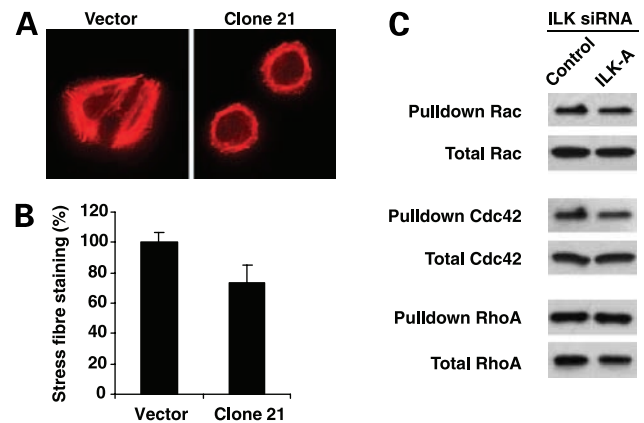


Figure 3. ILK regulates stress fiber formation and cell morphology in melanoma cells. **A**, representative images of stress fiber formation in vector and shRNA-stable clone 21. Cells seeded on coverslips were serum-starved overnight followed by serum stimulation with 10% fetal bovine serum for 1 h. The stress fiber staining was done as described in Materials and Methods. **B**, quantification of stress fiber staining intensity in **A**. Data was obtained from triplicate experiments. **C**, RhoA, Rac, and Cdc42 GTPase activity assay for transient ILK siRNA-transfected cells. MMRU cells were transfected with 25 nmol/L of either control siRNA or ILK siRNA. At 4 d posttransfection, the overnight serum-starved cells were analyzed for the activation of Rac, Rho, and Cdc42. Active Rac and Cdc42 were pulled down with GST-PBD, and active Rho was pulled down using GST-RBD. The bound Rac, Rho, and Cdc42 were detected by immunoblotting. Immunoblots of Rac, Rho, and Cdc42 from the corresponding whole cell lysate were used as the loading control.

and migration in mouse epithelial cells requires the activation of the Rho GTPase family members, Rac and Cdc42, in an ILK-dependent manner (22). We did not detect a significant difference in RhoA activation between control and ILK siRNA-transfected cells, but detected a 10% and 30% reduction in active GTP-bound Rac and Cdc42, respectively, in ILK knockdown cells (Fig. 3C). This agrees with the previous report that Rac and Cdc42, but not RhoA, are involved in ILK-mediated cell spreading and actin restructuring (22).

ILK Regulates Cell Attachment in Melanoma Cells

Because ILK is involved in actin organization in melanoma cells, we tested if ILK regulates cell attachment on fibronectin-coated plates. Cells were allowed to attach to fibronectin-coated plates for 1 h. The unattached cells were washed away. Attached cells were stained and quantified by crystal violet staining. Both ILK siRNA and shRNA treatment reduced the cell attachment ability of melanoma cells in fibronectin-coated plate by 61% and 45%, respectively (Fig. 4A and B).

ILK Regulates Cell Invasion and Anchorage-Independent Growth in Melanoma Cells

We next investigated the role of ILK in invasion and anchorage-independent growth of melanoma cells. We found that cells from ILK shRNA-stable clones 16 and 21 had a reduced ability to invade through Matrigel-coated Boyden chambers by 80% and 90%, respectively, when compared with the control cells (Fig. 5A). The invasive ability of cancer cells could be regulated through the

expression of MMPs. We did zymography assays to measure MMP activities in melanoma cells. As shown in Fig. 5B, there was no significant difference in MMP-2 activity between ILK shRNA-stable clone 21 and control cells, but MMP-9 activity was decreased in ILK knockdown cells by 50%. Our results are consistent with the previous report that ILK overexpression increases invasiveness in intestinal and mammary epithelial cells through increased expression of MMP-9 (23).

Transformed cells have the capacity to form anchorage-independent colonies in semisolid medium. To investigate if ILK plays an essential role in this transformed phenotype in melanoma cells, we did soft agar assays in ILK shRNA clones. We found that ILK shRNA-stable clones have a reduced anchorage-independent growth ability in soft agar. There were 50% and 70% fewer colonies formed in ILK shRNA-stable clones 16 and 21, respectively, when compared with the vector control (Fig. 5C and D).

ILK Is Important for Melanoma Tumor Growth *In vivo*

To further investigate the role of ILK in melanoma-genes, we asked if ILK was required for tumor growth *in vivo*. MMRU control and ILK shRNA-stable clones 16 and 21 were injected s.c. into severe combined immunodeficient mice and tumor growth was monitored. We found that tumor growth in ILK shRNA-stable clones 16 and 21 were drastically reduced by 90% and 80%, respectively, when compared with control cells (Fig. 6).

Discussion

Metastatic melanoma is a life-threatening disease with no effective treatment available. Therefore, it is important to understand the molecular mechanisms of melanoma progression in order to discover new targets to treat advanced melanoma. Our previous finding from tissue microarrays showed that ILK expression correlates with melanoma thickness and patient survival (2). As the thickness of the melanoma always associates with invasiveness, we tested if ILK was important in different processes involved in melanoma progression, including migration, cell attachment, invasion, anchorage-independent growth, and tumor growth *in vivo* by targeted ILK knockdown using siRNA or shRNA.

We first showed that ILK is important in melanoma cell migration (Fig. 1C and D), consistent with its role in the cell migration of other mammalian cells like HeLa, Chinese hamster ovary, and rat embryonic fibroblasts (24, 25). We also found that ILK knockdown severely affected the formation of the actin cytoskeleton and motility structures in melanoma cells (Fig. 3A). It has been shown that ILK overexpression induced the distribution of actin filaments onto the cell membrane to form these cell motility structure (26). ILK knockdown in mouse epithelial cell line *scp2* by antisense ILK expression delayed cell spreading and remodeling of actin cytoskeleton (22). Our results suggest that ILK plays an important role in melanoma cell migration through the regulation of actin cytoskeleton organization.

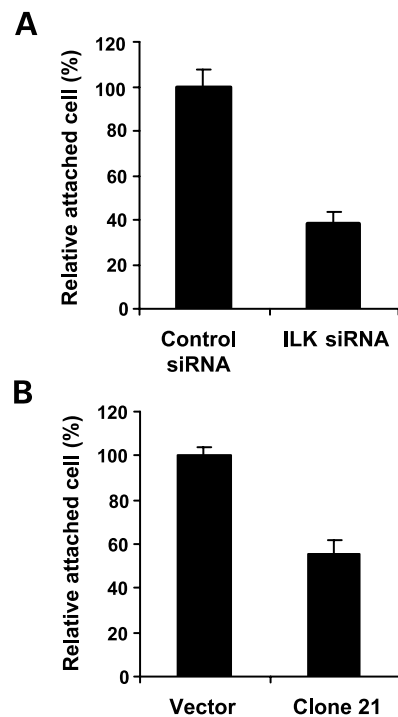


Figure 4. ILK regulates melanoma cell adhesion to fibronectin. Cell attachment assays were done as described in Materials and Methods for transient ILK siRNA treatment (A) or ILK shRNA-stable clone 21 cells (B). Briefly, cells were trypsinized and seeded on non-tissue culture plates coated with fibronectin for 1 h at 37°C. Unattached cells were washed away and attached cells were fixed and stained with crystal violet followed by solubilization of the dye and absorbance at 590 nm was measured. Data was obtained from triplicate experiments.

Actin organization is regulated through the Rho GTPase family proteins. We showed that Rac and Cdc42 activities, but not RhoA, were decreased in ILK knockdown cells. Rho activation leads to the assembly of stress fibers (27), whereas Rac is responsible for the production of lamellipodia and membrane ruffles, and Cdc42 is important for the formation of filopodia (28). We showed that stress fiber formation in ILK knockdown cells only decreased by 20% with no apparent change in RhoA activation, whereas the change in the cell morphology and the formation of motility structure in ILK knockdown cells were more dramatic with a reduction in Rac and Cdc42 activation. The pattern of activation of these Rho GTPase family members echoes with the organization of actin cytoskeleton after ILK knockdown. This implicates that ILK regulates the actin cytoskeleton through Rho GTPase family members, Rac and Cdc42, in melanoma cells. However, the reduction in Rac and Cdc42 activation in this assay was not as drastic as in other systems (22), especially because Rac activity was only minimally inhibited in MMRU cells after ILK knockdown, suggesting that Rac and Cdc42 may not be the only regulators of actin cytoskeleton reorganization in melanoma cells. Further study is needed to clarify other possible molecules through which ILK regulates cell migration in melanoma cells.

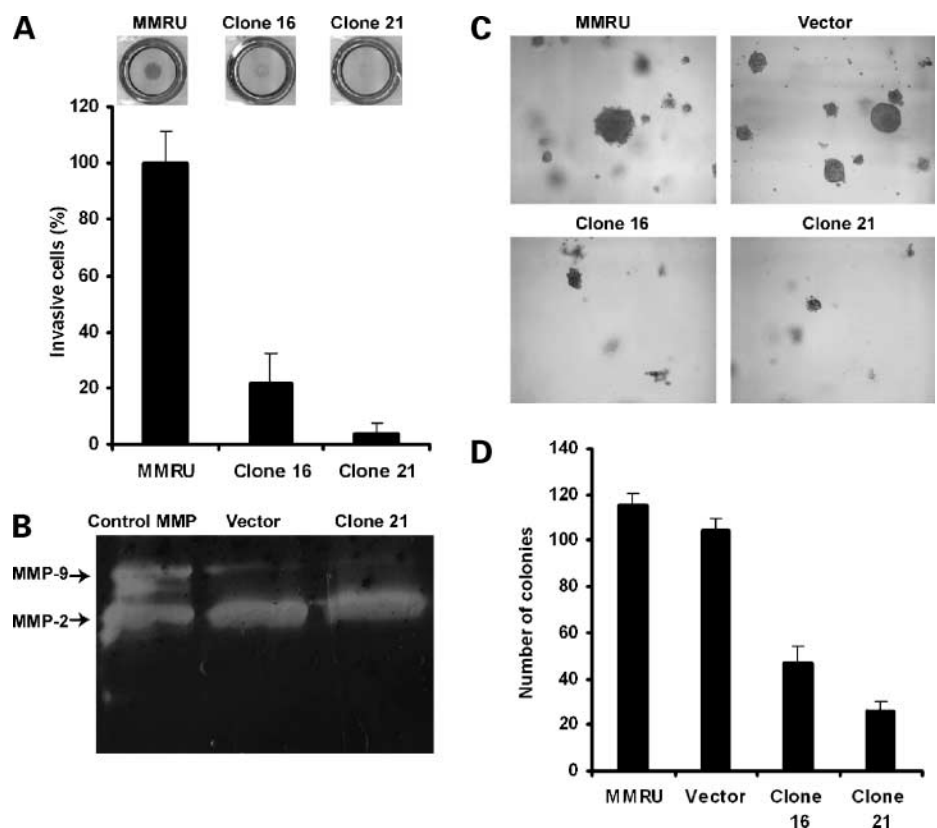
We showed that ILK is important for the early events in cell spreading and actin organization. We then further showed that ILK is important for melanoma cells to attach to fibronectin-coated plate. Upon engagement with the ECM, numerous signaling proteins are recruited to the adhesion sites in which cell-matrix contact establishes (29). Fibronectin is one of the major components in the ECM that cells bind through the extracellular domain of integrins. Integrin expression was shown to be important for melanoma cell attachment and migration on ECM proteins (30). Our results imply that ILK plays a central role in transducing signals when melanoma cells engage with the ECM regulating cell attachment. This is an important biological activity that contributes to cancer metastasis.

We found that ILK is important for melanoma invasion, which is consistent with the reports by Troussard et al. who showed that ILK overexpression increases invasiveness in intestinal and mammary epithelial cells through increased expression of MMP-9 via GSK-3 β and activator protein transcription factor (16, 23). The overall activity of MMPs is to produce a microenvironment suitable for tumor progression by various pathways (31). It has been shown in other studies that MMP-9 expression is important for melanoma progression (32). It has been suggested that MMP-9 is expressed or could only be induced in cell lines derived from advanced primary melanomas but not from

early stage primary lesions (33). Immunohistochemical study of the expression of MMP-9 in benign and malignant melanocytic lesions revealed that MMP-9 expression was exclusively expressed in the vertical growth phase, suggesting that MMP-9 is involved in early events in the degradation of ECM and invasion of melanoma cells (34). Our previous tissue microarray data shows that ILK expression correlates with melanoma thickness (2), which suggests a possible role for ILK to regulate melanoma invasion through MMP-9 expression when melanoma progresses. Various agents have been used to inhibit melanoma invasion through the inhibition of MMP-9 expression (35–38), possibly through inhibition of c-Jun phosphorylation at Ser^{63/73} (35, 38). ILK increases c-Jun containing activator protein activity and stimulates MMP-9 promoter activity and expression (16, 23); however, the phosphorylation of c-Jun at Ser⁷³ is not changed. The authors suggested that ILK-mediated activation of activator protein activity is through the inhibition of GSK-3 activity and phosphorylation of c-Jun by GSK-3 at a region proximal to the DNA-binding domain at amino acids 247 to 263. Such phosphorylation in c-Jun inhibits the activator protein activity (39).

In this study, we showed that ILK knockdown impaired the anchorage-independent growth of melanoma cells. It has been suggested that constitutive activation of ILK promotes anchorage-independent cell survival, possibly

Figure 5. ILK regulates melanoma cell invasion, MMP-9 activity, and anchorage-independent growth. **A**, cell invasion assay on ILK shRNA-stable clones. *Top*, representative images of invaded cells in the insets of Transwell chambers. *Bottom*, quantification of cell invasion. *Columns*, means from three independent experiments; *bars*, SD. **B**, MMP-9 activity is reduced in ILK shRNA clone 21. Conditioned media were collected in control or ILK shRNA-stable clone 21 cells and concentrated with centricon for 4 h. Recombinant MMPs and the same amount of protein from each sample were loaded in a 10% polyacrylamide gel containing 0.1% gelatin for the zymography assay. Gelatinolytic activity appears as a clear zone in the gel. **C** and **D**, soft agar colony formation assay for ILK shRNA-stable clones. Cell suspensions of parental MMRU or MMRU stable cells containing shRNA vector or ILK shRNA in 0.3% agar were overlaid on top of a 0.5% agar bed. **D**, after 15 d, colonies were photographed as in **C** (magnification, $\times 32$), and were counted under a light microscope. *Columns*, means from three independent experiments; *bars*, SD.



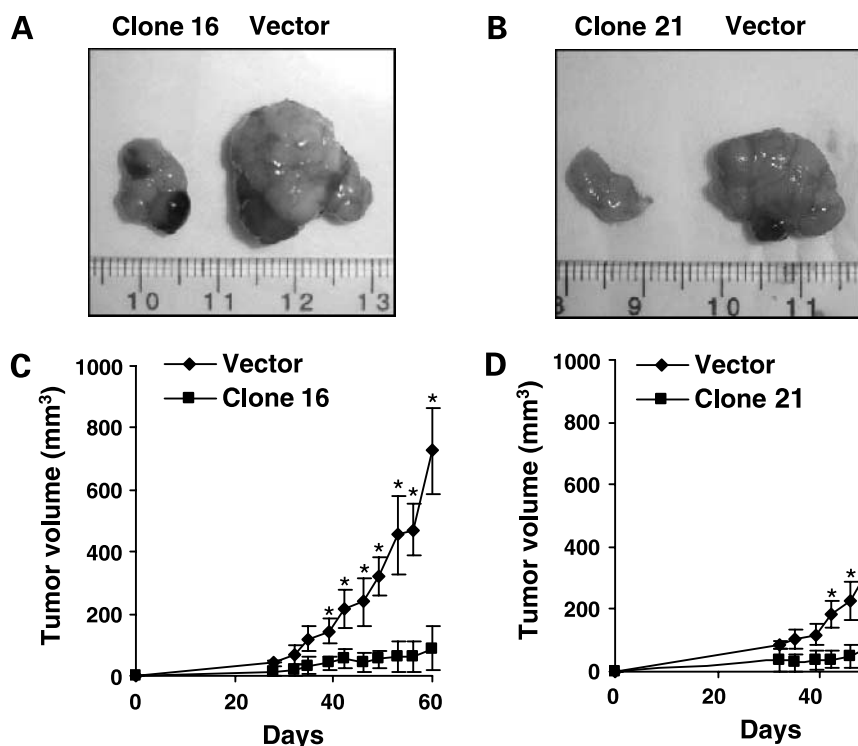


Figure 6. ILK shRNA inhibits the growth of melanoma xenografts in severe combined immunodeficient mice. **A** and **B**, images of the tumors excised from the severe combined immunodeficient mice s.c. injected with control or ILK shRNA-stable clone 16 (**A**) or ILK shRNA-stable clone 21 (**B**). **C** and **D**, estimated tumor volume of the control and ILK shRNA-stable clones 16 ($n = 5$) or clone 21 tumors ($n = 6$). *, $P < 0.005$.

through the suppression of apoptosis and anoikis by ILK-mediated activation of Akt/PKB and suppression of caspase-3 activation (6, 9). Our data indicate that ILK is also important for anchorage-independent cell survival in melanoma cells.

Because we showed that ILK is important in different processes involved in melanoma progression including cell migration, cell attachment, invasion, and anchorage-independent growth, we asked if ILK would affect overall tumor growth *in vivo*. We found that ILK knockdown impaired the growth of melanoma tumor xenograft *in vivo*. It should be noted that ILK knockdown did not affect cell proliferation *in vitro* (Fig. 1). It has also been shown in other studies that cell proliferation *in vitro* does not necessarily correlate with tumor growth *in vivo*. For instance, psoriasin regulates breast cancer cell migration and invasion without affecting cell proliferation *in vitro* but influences tumor growth *in vivo* (40, 41). Tissue inhibitor of metalloproteinase-1 modulation does not affect the cell proliferation of pancreatic cancer cells *in vitro* but attenuates tumor growth *in vivo* (41). Therefore, the inhibition of melanoma growth by ILK knockdown *in vivo* was most likely due to the inhibition of cell migration, cell attachment, invasion, and anchorage-independent growth rather than cell proliferation.

Because ILK is up-regulated in several types of cancers, such as colon, breast, prostate, non-small cell lung cancer, as well as in melanoma, and is often associated with the progression of cancer (6, 42–44), it may be used as an

attractive molecular target for cancer therapy. In this study, we showed that ILK regulates melanoma cell migration, invasion, anchorage-independent growth, and tumor growth *in vivo*. ILK knockdown by RNA interference impaired all of the above processes involved in melanoma progression. Therefore, this study provides the biological basis for inhibiting ILK by RNAi as a novel therapeutic approach for melanoma. Efforts have been made to inhibit tumor growth *in vivo* by inhibiting ILK activity. For instance, the inhibition of ILK kinase activity by pharmacologic ILK inhibitor QLT0267 suppresses tumor growth of anaplastic thyroid cancer by 50% to 60% (45). Another ILK inhibitor, QLT0254, inhibits the tumor growth of pancreatic cancer by 40% (46). In these studies, the inhibition of tumor growth *in vivo* is achieved by the inhibition of ILK kinase activity using small molecule inhibitors. In our study, we specifically knocked down ILK expression by shRNA and achieved 80% to 90% of inhibition in tumor growth. Therefore, there is a great potential to use ILK shRNA as gene therapy in melanoma. One advantage of using RNA interference in therapy is that once the RNA interference silencing complex is formed inside a cell, it is incredibly stable for up to a few weeks in some cell types (47). It should be noted that shRNA sequences targeting different regions of the ILK show different efficacies in ILK knockdown. We tested four sequences for construction of shRNA targeting ILK genes at positions 472 to 492, 952 to 962, 1034 to 1044, and 1107 to 1117, and the sequence 472 to 492 showed the highest efficacy in knocking down ILK

expression (data not shown). Therefore, we propose that this shRNA targeting the ILK sequence 472 to 492 may be applied in cancer gene therapy in melanoma, and possibly other forms of human cancers with elevated ILK activity.

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