Targeting Integrin-Linked Kinase Suppresses Invasion and Metastasis through Downregulation of Epithelial-to-Mesenchymal Transition in Renal Cell Carcinoma

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Abstract

Renal cell carcinoma (RCC) is the most common malignancy in the kidney. Antiangiogenic targeted therapies inhibit the progression of RCC, but have limited impacts on invasion or metastasis of tumor cells. Integrin-linked kinase (ILK) is a serine/threonine kinase implicated in the regulation of cell growth/survival, cell-cycle progression, epithelial–mesenchymal transition (EMT), invasion/migration, and angiogenesis. However, the role of ILK in RCC has not been evaluated. We investigated the role of ILK on cancer progression and metastasis and the therapeutic potential of ILK inhibition in RCC. Our investigation reveals that ILK is expressed at a low level in normal cells and low-stage RCC cells and is highly expressed in advanced and metastatic cells. Caki-1, a metastatic RCC cell line, showed higher expression of molecular EMT markers, including Snail and Zeb1, but decreased activity of GSK3β. Knockdown of ILK using small interference (si)-ILK minimally inhibited tumor proliferation and cell-cycle progression was not significantly affected. However, ILK knockdown suppressed the formation of stress fibers and focal adhesions and impeded phenotypic EMT markers, including cell migration and invasion, in Caki-1 and UMRC-3 cells. Finally, in vivo knockdown of ILK suppressed the progression, invasion, and metastasis of primary RCC in nude mice by downregulation of EMT markers (Snail, Zeb1, vimentin, and E-cadherin). Our results show that ILK may be essential for invasion and metastasis in RCC and regulates vimentin and E-cadherin expression by regulating the EMT-related transcription factors Snail and Zeb1. These results suggest that ILK may be a potential target in RCC. Mol Cancer Ther; 14(4); 1024–34. ©2015 AACR.

Introduction

Renal cell carcinoma (RCC) is the most common renal malignancy, and the incidence and mortality rates have been increasing steadily worldwide at a rate of 2% to 3% per decade (1, 2). Despite an overall shift in earlier diagnosis of RCC, approximately 20% to 30% of all patients are diagnosed with metastatic disease; even with surgical extirpation, 20% of patients will relapse and develop metastasis during follow-up (3). Antiangiogenic therapies targeting vascular endothelial growth factor (VEGF) have shown clinical benefits in patients with advanced RCC, but the results are generally thought to be cytostatic and do not cure patients. These treatments effectively inhibit tumor progression through deprivation of oxygen and nutrition from the tumor microenvironment, but cannot block metastasis of RCC cells. Moreover, there is growing evidence that antiangiogenic therapies can accelerate invasion and metastasis by making the tumor microenvironment more fertile (4, 5).

Epithelial–mesenchymal transition (EMT), defined as the loss of epithelial characteristics and the acquisition of a mesenchymal phenotype, is now known to also occur in a variety of disease states, including during cancer progression (6, 7). EMT is characterized by a combined loss of epithelial cell junction proteins, such as E-cadherin, and the gain of mesenchymal markers, such as vimentin, and is believed to play an essential role in tumor invasion and metastasis. A number of distinct molecular processes are engaged to initiate an EMT and allow it to reach completion (8). These include activation of transcription factors, expression of specific cell-surface proteins, reorganization and expression of cytoskeletal proteins, and the production of extracellular matrix (ECM)–degrading enzymes (8). Upon activation, EMT-inducing transcription factors, including Snail, Slug, zinc finger E-box binding homeobox 1 (Zeb1), and Twist, act pleiotropically to choreograph the mesenchymal transition (9). Implementation of EMT by these cells depends on cell-surface proteins such as β4 integrins, α5β1 integrin, and αVβ6 integrin (9, 10). Activation of EMT programs is also facilitated by the disruption of cell–cell adherence junctions and integrin-mediated cell–ECM adhesions (11–13).

Integrins are cell-surface proteins representing the main class of receptors for ECM proteins, such as collagen and laminin, and are located in the basement membrane. Attachment to, and movement along, the ECM is often integrin-dependent. Integrin-linked
Integrin-Linked Kinase in RCC

kinase (ILK), originally identified as a β1-integrin subunit cytoplasmic domain interactor, is a widely expressed serine/threonine protein kinase located in focal adhesions (14). ILK is an essential protein that connects integrins to the actin cytoskeleton and regulates actin polymerization. Localization of ILK induces focal adhesion plaques and coordinates cell spreading and actin organization. These adhesion sites are major intracellular signaling centers where ILK plays a central role in transducing many of the biochemical signals initiated by cell–matrix interactions that regulate fundamental processes such as growth, proliferation, survival, differentiation, migration, invasion, and angiogenesis (15–19).

Overexpression of ILK is often a prominent feature of human malignancies and its increased abundance in tumor tissues correlates with poor outcome (20–24). Recent reports suggest that overexpression of ILK in epithelial cells induces the EMT by repressing E-cadherin expression, activating nuclear β-catenin, and inducing a transformed, tumorigenic phenotype (25). However, the role of ILK in RCC cell survival, invasion, and metastasis is unclear. Here, we show that, in metastatic RCC cells, both ILK and its downstream effectors related to the EMT are highly expressed. Overexpression of ILK increased tumor cell activity for migration and invasion. Finally, we show that ILK is essential for invasion and metastasis of RCC both in vitro and in vivo, and demonstrate its potential as a therapeutic target as antimetastasis therapy in RCC.

Materials and Methods

Cell culture and reagents

The human RCC cell line Caki-1 was obtained from the ATCC and maintained in McCoy 5A Medium (HyClone, Thermo Fisher Scientific) supplemented with 10% fetal bovine serum (FBS) in 2011. Two additional human RCC cell lines, UMRC-3 and UMRC-6, were gifts from Dr. P. Black (Vancouver Prostate Centre, University of British Columbia, Vancouver, BC, Canada) in 2011 and maintained in minimal essential medium (MEM; Invitrogen) supplemented with 10% FBS and 2 mmol/L-glutamine. Cell line authentication for UMRC-3 and UMRC-6 from Dr. P Black was performed using the fingerprinting by an AmpliFISTR identifier amplification kit (Applied Biosystems) in Korean Cell Line Bank, Korea. The human RCC cell line 786-O was purchased from the ATCC and maintained in RPMI-1640 Medium (HyClone, Thermo Fisher Scientific) supplemented with 10% FBS in 2011. The human renal epithelial cell line HK-2 was purchased from the ATCC and cultured in DMEM/Ham F12 (Invitrogen) supplemented with 10% FBS and 2 mmol/L-glutamine in 2012. All cells were cultured at 37°C in a humidified atmosphere with 5% CO2. For all experiments, cell lines were maintained for no more than 2 months.

Small-interfering RNAs

Caki-1 and UMRC-3 cells were transiently transfected with 21-nt siRNA molecules targeting the integrin-binding domain of ILK (5’T-GAGGAGTACCATCAGCAGAATGTA-3’) or a nonsilencing sequence siRNA (si-Scr). Caki-1 and UMRC-3 cells were plated onto 6-well plates at a density of 1.2 × 105/well and then transfected at 30% to 50% confluence for 16 hours with siRNAs by Lipofectamine 2000 (Invitrogen Life Technologies) diluted with OPTIMEM (Invitrogen Life Technologies) according to the manufacturer’s instructions. After transfection, media was replaced and cells were then incubated for 48 to 72 hours based on the purpose of the experiment.

Stable transfections

To generate ILK-overexpressing RCC cells, UMRC-6 cells were transfected with either pcDNA3.1 His-V5–tagged kinase constitutively active ILK (ILK S343D), expressing constitutively kinase-active mutant ILK or pcDNA 3.1 His-V5 as a control (mock; provided by Dr. Shouket Dedhar, BC Cancer Agency, Vancouver, BC, Canada; ref. 26). Cells grown in 6-cm dishes were transfected for 4 hours with 3-μg DNA using a 1:3 ratio of Lipofectamine 2000 transfection reagent (Invitrogen) according to the manufacturer’s recommendations (26). Clones were selected with G418 (Invitrogen). For stable knockdown of ILK in Caki-1 cells, oligonucleotides for ILK small hairpin RNA (shRNA; 5’-CCGGCACTGACAACCATCACGCTCAGCAGCAGAATGTA-3’) were purchased from Sigma-Aldrich. To generate lentiviral particles, the pLKO.1-puro ILK1 shRNA plasmid was cotransfected with the gal/pol plasmid pMDLg/pRRE, envelop plasmid pRSV-REV and pMD2.G by Lipofectamine 2000 reagent (Invitrogen) in the human embryonic kidney cell line 293FT transfected with SV40 large T antigen. After 48 hours, the resulting supernatant was collected, filtered through a 0.45-μm syringe-driven filter unit (Sartorius Stedim Biotech), and infected with 1 μg/mL polybrene (hexadimethrine bromide; Sigma-Aldrich) into Caki-1 cells. After incubation with virus supernatant for 72 hours, cells were selected with 1 μg/mL puromycin (Sigma-Aldrich). pLKO.1-puro nonsilencing shRNA was used as a control. Similarly, to develop the orthotopic renal tumor model (Ckai-1-luc), Caki-1 cells were stably transduced with lentiviral particles containing the firefly luciferase gene. The infectious virus was generated by cotransfection of 293FT cells with the FULLBW vector [derived from FUGW (a generous gift from David Baltimore, California Institute of Technology, Pasadena, CA), but GFP was replaced with the firefly luciferase gene using the Invitrogen Gateway System] and the packaging plasmids pCMVDR8.2 and pMD.G. After lentivirus transduction, Caki-1 cells were further selected with blasticidin (Invitrogen). Stable cell clones were then confirmed by Western blot analysis. After selection and confirmation, stable cell lines were frozen at early (less than 10) passages.

Cell viability assay

Cells were seeded onto 6-well plates at a density of 1.2 × 105 per well and incubated overnight. After transfection, cells were incubated for 72 hours and then fixed with 1% glutaraldehyde and stained with 0.5% crystal violet solution. Cells were washed with water and the remaining crystal violet was resolved with Sorensen’s solution. Absorbance was measured at 562 nm by spectrophotometry. All experiments were performed in triplicate.

Cell migration assay

A wound-healing assay was used to assess directional cell migration. Cells were plated onto 6-well plates and allowed to form a confluent cell monolayer. Wounds were made in each well using a 200-μl pipette tip. After the scratch, floating cells and debris were washed out with phosphate-buffered saline (PBS) twice and cells were then incubated in culture media for an additional 36 hours. Wound-healing was recorded every 6 hours by microscopy. Each experiment was performed in triplicate and, in each experiment, the area of cells that migrated across the
were analyzed on a FACSCanto II (20 g/mL; Sigma Chemical) for 30 minutes. Relative DNA contents were determined using the TUNEL assay software (BD Bioscience).

**Cell invasion assay**

Matrigel-coated inserts in 24-well plates (BD Matrigel Invasion Chamber, 8.0-μm PET membrane) were used to assay cell invasion. A cell suspension containing 1 × 10⁶ cells/mL in serum-free media was added to the inside of the insert. Lower wells were filled with 500-μL media containing 10% FBS. Plates were incubated for 24 or 48 hours in a cell culture incubator. Nonmigratory cells on the top of the membrane were carefully removed using the ends of cotton-tipped swabs three times. The remaining migratory cells were stained with 0.5% crystal violet solution, gently washed several times with water, and allowed to air dry. Each experiment was performed in triplicate. Quantification was performed by counting migratory cells using light microscopy in three individual fields per insert.

**Cell-cycle analysis**

Cells were harvested and fixed with 70% alcohol as a single-cell suspension. The fixed cells were collected by centrifugation and washed with PBS. The cells were incubated with RNase A (1 mg/mL) for 30 minutes at 37°C and then incubated with propidium iodide (20 g/mL; Sigma Chemical) for 30 minutes. Relative DNA contents were analyzed on a FACSCanto II flow cytometer and FACSDiva software (BD Bioscience).

**TUNEL assay**

Cells were fixed with 4% paraformaldehyde after 48 hours of treatment and stained using a terminal deoxynucleotidyl transferase–mediated diUTP nick end labeling (TUNEL) kit (Promega). Apoptotic (fluorescent green) and total cells were counted under a fluorescence microscope and data were recorded. Images were obtained using a confocal microscope (LSM Meta 700, Carl Zeiss).

**Antibodies and reagents**

Anti-ILK (C-19), anti-vinculin, and anti-actin antibodies were purchased from Santa Cruz Biotechnology. Anti-pGSK3b, anti-Snail, anti-Zeb1, and anti-vimentin antibodies were purchased from Cell Signaling Technology. Anti–E-cadherin antibody was purchased from BD Biosciences. Anti-goat, anti-rabbit, and antimouse IgG secondary antibodies were used (1:5,000) for Western blot analysis or immunocytochemical staining.

**Western blot analysis**

Proteins were harvested in RIPA lysis buffer containing vandate, phosphatase inhibitor, and phenylmethylsulfonylfluoride (PMSF). Sodium dodecyl sulfate–polyacrylamide gel electrophoresis (SDS-PAGE) was performed, and gels were then transferred to a nitrocellulose membrane using the trans-blot system (Bio-Rad) as described previously (26). Membranes were blocked in 5% skim milk diluted with Tris-buffered saline containing Tween-20 (TBST) for 1 hour at room temperature and then immunoblotted with primary antibodies diluted in 3% skim milk diluted with TBST for 1 hour at room temperature or overnight at 4°C. Amersham (GE Healthcare) or Supersignal (Thermo Scientific) was used as Western blotting detection reagents.

**Immunocytochemistry and confocal microscopy**

To assess the expression of ILK in cells, cells were plated onto glass coverslips in 6-well plates overnight to allow cell attachment, washed with ice-cold PBS, fixed with ice-cold methanol mixed with acetone (3:1) for 10 minutes at room temperature, and treated with 0.25% Triton X-100 in PBS solution for 10 minutes. After blocking with 3% bovine serum albumin for 30 minutes, cells were incubated with anti-ILK at room temperature for 1 hour. Cells were washed three times with TBS and incubated with Alexa Fluor 488 anti-goat antibody for 1 hour in a dark room. Nuclei were stained with DAPI. To evaluate the expression of ILK, Snail, Zeb1, vimentin, E-cadherin, actin, vinculin, and Ki-67 in cells after ILK knockdown, cells were processed in a similar manner with anti-actin and -vinculin antibodies. Immunofluorescence was visualized using a Zeiss confocal laser-scanning microscope.

**Orthotopic xenograft model of human RCC**

All animal experiments were conducted in compliance with accepted standards of the University of British Columbia Committee on Animal Care. Six- to 8-week-old female nude mice (nu/nu) were anesthetized with isoflurane. To develop the orthotopic renal tumor model (Caki-1 cells) the right kidney was exposed via a small incision in the flank area and a suspension of 1 × 10⁶ Caki-1-luc cells in 50-μL PBS was injected into the subcapsular area of the exposed kidney through a 30-gauge needle. The punctured site was pressed by a cotton-tipped swab until bleeding ceased. The kidney was returned to the normal position and the flank incision was closed with 6-0 vicryl sutures. Orthotopic growth of RCC was confirmed via bioluminescence. Invasion and metastasis were also traced using bioluminescence. Animals were imaged every week using an IVIS2000 Imaging system (Caliper Life Sciences). Following treatment, mice were euthanized by a CO₂ chamber and cervical dislocation. All tumors were fixed in 10% formalin for immunohistochemical staining or frozen at −80°C for protein and RNA analysis.

**Immunohistochemistry**

All tissues were fixed in 10% neutral buffered formalin and embedded in paraffin using standard surgical pathology protocols. Tissue sections (5 μm) were dewaxed and antigen retrieval was performed in citrate buffer (pH 6) using an electric pressure cooker set at 120°C for 5 minutes. Sections were incubated for 5 minutes in 3% hydrogen peroxide to quench endogenous tissue peroxidase. Staining was performed using the Ventana Discover XT Autostainer (Ventana Medical Systems) with a biotin streptavidin system and a solvent-resistant DAB Map kit. Primary monoclonal antibodies were directed against ILK (Santa Cruz Biotechnology), snail (Santa Cruz Biotechnology), Zeb1 (Abcam), and vimentin (Abcam), which were diluted with PBS at a ratio of 1:100. All tissue sections were counterstained with hematoxylin, dehydrated, and mounted. The Meta Morph 4.6 software was used for computerized quantification of immunostained target proteins.

**Statistical analysis**

All observations were confirmed by at least three independent experiments. The results are presented as the mean ± SE. Statistical analysis was performed using SigmaPlot 12 (Systat Software Inc.). The Student t test was used for two-group comparisons. Differences were considered significant when P values were 0.05 or less.
Results

ILK is highly expressed in advanced RCC

To investigate the expression of ILK in RCC, we first examined baseline expression of ILK by immunofluorescence using a panel of human RCC cell lines and normal human renal epithelial cells. Noncancerous human renal epithelial cells (HK-2) poorly expressed ILK and RCC cell lines generally showed higher expression of ILK compared with normal renal cells (Fig. 1A). In addition, ILK was more highly expressed in cell lines derived from high-stage tumors (UMRC-3 from a stage IV primary tumor and Caki-1 from a metastatic tumor) compared with a cell line from a low-stage tumor (UMRC-6 from a stage I primary tumor), suggesting that ILK may be related to the aggressive features of high-stage RCC (Fig. 1B).

ILK expression is correlated with the expression of EMT markers in RCC

Markers related to the EMT were assessed using a panel of human RCC cell lines and normal human renal epithelial cells. Phosphorylation of GSK3β was low in Caki-1 cells. ILK expression was correlated with the expression of transcription factors Snail and Zeb1, as well as vimentin. E-cadherin was detected in HK-2 cells, but not in all RCC cell lines (Fig. 1B). We confirmed this correlation, shown with the Western blot analyses, using immunofluorescence confocal imaging (Supplementary Fig. S1A–S1E). These results show that ILK expression is related to EMT markers in various RCC cells.

Overexpression of ILK induces phenotypic changes by upregulating Snail and Zeb-1 in RCC

We next developed ILK-overexpressing UMRC-6 cells to assess the role of ILK expression in RCC. UMRC-6 cells have low ILK expression compared with other RCC cell lines, but ILK-overexpressing UMRC-6 cells demonstrated high expression of ILK compared with mock cells (Fig. 2B). In vitro, ILK-overexpressing cells showed a more fibroblast-like morphology and loss of polarization and contact inhibition, suggesting a morphologic transition to a mesenchymal phenotype by ILK overexpression. To determine whether these morphologic changes are related to the EMT, we assayed EMT markers in ILK-overexpressing UMRC-6 cells (Fig 2A). Western blot analysis confirmed that ILK overexpression was associated with the expression of Zeb-1 and Snail, critical transcription factors in the EMT, and also vimentin, a major cytoskeletal component of mesenchymal cells (Fig. 2B). EMT characteristics, migration, and invasion, as well as a morphologic change, were evaluated using a wound-healing assay and Matrigel invasion assay. These assays confirmed that ILK overexpression increased the migration and invasion potential in RCC cells (Fig 2C–F). In summary, these results suggest that ILK induces the EMT through upregulation of Zeb-1 and Snail in RCC cells.

Cell survival is not significantly regulated by ILK in RCC

To evaluate the effects of ILK on tumor growth and cell-cycle progression in RCC, we transiently knocked down ILK in RCC cells using RNA interference targeting ILK. siILK successfully inhibited ILK expression at a concentration of 40 nmol (Fig. 3A). However, a cell growth assay using HK-2, UMRC-6, UMRC-3, and Caki-1 cells showed that in vitro proliferation of RCC cells was not suppressed by ILK knockdown compared with the normal renal epithelial cell line HK-2 (≤20%; Fig. 3B). Also, the insufficient inhibition of proliferation was unaffected by increasing siRNA concentration in Caki-1 cells (Fig. 3C). Cell-cycle analysis showed that ILK knockdown did not induce apoptosis or significant cell-cycle arrest in Caki-1 cells. In summary, ILK plays a minimal role in cell survival and cell-cycle pathways in RCC cells. We confirmed the minimal effect of ILK knockdown on cell proliferation and apoptosis using a TUNEL assay and Ki-67 staining (Fig. 3E and F).

Knockdown of ILK suppresses migration and invasion in RCC

To study the effect of ILK inhibition on tumor migration in RCC cells, we used a wound-healing assay after transient knockdown of ILK in HK-2, UMRC-6, UMRC-3, and Caki-1 cells expressing UMRC-6 cells demonstrated high expression of ILK compared with mock cells (Fig. 2B). In vitro, ILK-overexpressing cells showed a more fibroblast-like morphology and loss of polarization and contact inhibition, suggesting a morphologic transition to a mesenchymal phenotype by ILK overexpression. To determine whether these morphologic changes are related to the EMT, we assayed EMT markers in ILK-overexpressing UMRC-6 cells (Fig 2A). Western blot analysis confirmed that ILK overexpression was associated with the expression of Zeb-1 and Snail, critical transcription factors in the EMT, and also vimentin, a major cytoskeletal component of mesenchymal cells (Fig. 2B). EMT characteristics, migration, and invasion, as well as a morphologic change, were evaluated using a wound-healing assay and Matrigel invasion assay. These assays confirmed that ILK overexpression increased the migration and invasion potential in RCC cells (Fig 2C–F). In summary, these results suggest that ILK induces the EMT through upregulation of Zeb-1 and Snail in RCC cells.
ILK in RCC cells using siILK. The wound-healing assay confirmed that cell migration into the wound was delayed significantly by ILK knockdown in Caki-1 cells (Fig. 4A and B). Cell growth assay and Ki-67 staining suggested that ILK has minimal effects on the proliferation of RCC cells (Fig. 3), but to completely rule out the effect of cell proliferation on migration, the wound-healing assay was repeated after mytomycin C treatment. The results confirmed that ILK knockdown suppressed the migration of RCC cells (Fig. 4C and D). We also assessed cell invasion using a Matrigel invasion assay, which showed that invasion through the Matrigel pore was reduced by ILK knockdown in Caki-1 and UMRC-3 cells (Fig. 4E–G). Immunocytochemical staining showed that E-cadherin expression was induced by knockdown of ILK (Fig. 4H). These results suggest that the invasive potential of RCC can be suppressed by ILK inhibition.

ILK knockdown impedes cell attachment by regulating stress fiber formation and focal adhesion in RCC

After transient knockdown of ILK, cells exhibited less attachment to cell culture plates (Fig. 5A). From this finding, we hypothesized that ILK knockdown affects both cell attachment and movement by changing cytoskeleton organization. Then, we tested if knockdown of ILK reduces cell attachment to fibronectin-coated plates. ILK knockdown by siILK impeded the attachment of RCC cells (Fig. 5B). Rhodamine-phalloidin staining showed that ILK knockdown impeded stress fiber formation and organization compared with control cells (Fig. 5C and D). To evaluate actin filament organization and focal adhesions, immunofluorescence staining using anti-actin and vinculin antibodies in UMRC-3 cells replated after transient knockdown of ILK was used. ILK knockdown significantly reduced actin filaments and organization and reduced focal adhesions (Fig. 5E). These results suggest that ILK plays an important role in stress fiber formation and focal adhesion in RCC cells, and inhibition of ILK impedes cell attachment by suppressing the organization of stress fibers.

ILK is necessary for invasion and metastasis in an orthotopic model of human RCC

To investigate the role of ILK in in vivo tumor invasion and metastasis, we created an orthotopic RCC mouse model by inoculating stably transfected ILK knockdown luciferase-expressing Caki-1 cells into the subscapular renal parenchyma of nude

Figure 2. Morphologic and molecular changes after stable transfection with ILK in UMRC-6 cells (low-stage RCC cells). A, morphologic changes in UMRC-6 cells after stable transfection with ILK. When cells reached confluence, photographs were obtained using a light microscope at ×20 magnification. B, changes in the expression of EMT markers after ILK overexpression. Whole-cell lysates from UMRC-6 cells transfected with pcDNA3.1 His-V5-tagged kinase constitutively active ILK (ILK S343D) expressing constitutively kinase-active mutant ILK or vector control were subjected to Western blotting to examine the expression of ILK and EMT markers. β-Actin was used as a loading control. C and D, effect of ILK overexpression on migration of UMRC-6 cells. When cells reached confluence, a wound was made by scratching and the percentage of wound-healing was calculated. The photos were taken at ×20 magnification. E and F, effect of ILK overexpression on the invasion of UMRC-6 cells. A cell suspension was plated on Matrigel-coated insert plates and migratory cells were stained with 0.5% crystal violet solution and counted under a light microscope. The photos were taken at ×20 magnification.

**P < 0.05 vs. mock.
mice (Fig. 6A). The growth rate of the primary tumors inoculated into the kidney was much lower in shILK transfectants than in vector control transfectants (Fig. 6B and C). In addition, it was clear that invasion and metastasis were reduced in animals inoculated with shILK transfectants compared with vector control transfectants. Direct invasion of renal tumors into adjacent tissues or organs, including perirenal tissues, intestines, liver, and abdominal muscles, and disseminated seeding nodules in the intestine, spleen, and peritoneum were found in xenografts expressing mock shRNA, whereas no invasion or metastasis was observed and only intrarenal tumors were found in xenografts expressing shILK (Fig. 6D). Immunohistochemical staining showed that ILK was successfully knocked down in vivo, and also that Snail, Zeb-1, and vimentin were downregulated and...
E-cadherin was upregulated by ILK knockdown (Fig. 6E and F). These results suggest that ILK is essential for invasion and metastasis in RCC, and also show that ILK may serve as a therapeutic target for antimetastasis therapy of RCC.

In vitro knockdown of ILK had minimal effect on cell proliferation in RCC cells, whereas in vivo knockdown of ILK showed marked inhibition of primary orthotopic tumor growth, as well as of invasion and metastasis. To confirm this, we evaluated the antiangiogenic effect of ILK using immunohistochemical staining and confirmed significant suppression of tumor angiogenesis by ILK knockdown in RCC xenografts (Supplementary Fig. S2).

Discussion

The development of novel therapies that suppress cancer metastasis is an urgent therapeutic need in RCC, as most existing approved therapies for metastatic disease have been documented to prevent disease progression rather than clearly improve survival (27, 28). We have shown that ILK may be important in invasion and metastasis in advanced RCC. In addition, we showed that ILK might promote EMT by regulating the transcription factors Snail and ZEB1, as well as cell movement by inducing focal adhesions and actin organizations in RCC. Inhibition of ILK suppresses both migration and invasion in RCC by impeding the formation of stress fibers and focal adhesion plaques. Inhibition of the primary tumor was supported by significant suppression of tumor angiogenesis after ILK knockdown in RCC xenografts. Suppression of metastasis, as well as primary tumor size, in orthotopic renal xenografts is consistent with the hypothesis that ILK may serve as a therapeutic target for inhibiting invasion and metastasis in advanced RCC.

ILK plays a central role in cell signaling and is related to tumor proliferation and cell-cycle progression in many types of cancer, but the role of ILK in RCC is less clear. A recent study suggested a
possible role for ILK in the progression of RCC by showing that ILK expression is correlated with tumor severity in clear-cell RCC (29). In this study, the RCC cell line from a metastatic lesion showed the highest expression of ILK and displayed higher expression of EMT-related transcriptional factors Snail and Zeb1, which suggests that ILK might be related to the metastatic potential of RCC cells. Although our study showed that ILK is not a main driver of tumor proliferation according to in vitro experiments, marked suppression of primary tumor size was observed in orthotopic xenografts containing shILK transfectants, suggesting that inhibition of ILK may suppress tumor progression by blocking cell movement and angiogenesis rather than direct suppression of tumor proliferation in RCC.

Metastasis requires the interaction of tumor cells with the ECM, which is mediated by the formation of elongated, integrin β1-containing adhesion plaques (30). The development of such plaques requires the prior assembly of integrin β1-containing filopodium-like protrusions. Actin-rich protrusions morphologically resembling filopodia formed by cells growing in monolayer culture and knockdown of ILK reduced the number of filopodium-like protrusions in mouse mammary carcinoma cells with metastatic potential (30). We showed that ILK is essential for focal adhesions and actin organizations required for metastatic movement of RCC cells, and that inhibition of ILK suppresses the formation of focal adhesion plaques. In our study, ILK knockdown caused a reduction in focal adhesion plaques and stress fiber formation, and also changed the morphology of RCC cells from a spindle shape to a rounded shape, which is consistent with data showing reduced filopodium-like protrusions in mammary carcinoma cells. In summary, these results suggest that ILK plays a central role in the formation of focal adhesion plaques through reorganization of cytoskeletons for metastasis in RCC.

The key mechanism by which ILK regulates the EMT is through inhibition of transcription of E-cadherin and vimentin. Multiple signaling pathways can regulate the EMT through modulating Snail, Twist, and Zeb family transcription factors (31). Our study showed that, through the overexpression of ILK in cells derived from low-stage RCC, ILK expression induces vimentin through upregulating the expression of Snail and Zeb1 in RCC. Snail, a zinc-finger transcription factor, triggers the EMT process by...
repressing E-cadherin expression and is regulated by GSK-3β (32). Zeb-1 is the Zeb family zinc finger transcription factor. The expression of Zeb proteins in epithelial cells strongly inhibits CDH1 gene expression, and this mechanism has been implicated in the EMT, tumorigenesis, and metastasis. Zeb factors can also regulate the expression of various EMT- and tumor-related genes.
such as genes encoding proteins critical to maintain the epithelial phenotype, such as E-cadherin, plakophilin 2 and ZO3 (31). Although the mechanism underlying ILK-mediated regulation of Zeb-1 and Snail is not defined, it was noted that overexpression of ILK in epithelial cells increases the mRNA levels of both Zeb-1 and Snail (33). Also, the major ILK responsive element, SIRE (Snail ILK responsive element) is located in the −134 to −69 region of the Snail-1 promoter and ILK knockdown was shown to downregulate Snail-1 promoter activity and upregulate E-cadherin promoter activity (34). Because it is known that the transcription of Zeb-1 is regulated by Snail-1 (33), we propose that ILK indirectly regulates the transcription of Zeb-1 via Snail, though we cannot exclude the possibility that ILK also directly regulates Zeb-1. Likewise, PARP-1 was identified as a SIRE-binding protein. ILK silencing inhibited PARP-1 binding to SIRE, and PARP-1 silencing decreased both Zeb1 and Snail, which resulted in the upregulation of E-cadherin (35).

Vimentin, the intermediate filament protein, is an important marker of the EMT and a requisite regulator of mesenchymal cell migration (36). Recent studies showed that vimentin also contributes functionally to EMT phenotypes including cell migration and invasion. This suggests that ILK plays a critical role in directly regulating EMT signals, as well as mediating EMT signals to reorganization of the cytoskeleton in RCC cells (36, 37). Our data show that vimentin expression is regulated by ILK expression, which suggests that ILK regulates the EMT in RCC in various ways. In conclusion, our study shows that ILK regulates the EMT in RCC cells, and targeting ILK suppresses invasion and metastasis through inhibition of the EMT in RCC. These results suggest that ILK may serve as a target for antimetastasis therapy for RCC.

Disclosure of Potential Conflicts of Interest
No potential conflicts of interest were disclosed.

Authors’ Contributions
Conception and design: K.S. Han, M.E. Gleave, A.I. So
Development of methodology: K.S. Han, N. Li, P.A. Raven, M.E. Gleave, A.I. So
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Targeting Integrin-Linked Kinase Suppresses Invasion and Metastasis through Downregulation of Epithelial-to-Mesenchymal Transition in Renal Cell Carcinoma

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