α-Catenin Drives Metastasis by Activating ILK and Driving an ανβ3 Integrin Signaling Axis

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Abstract

α-Catenin is an oncoprotein that helps sustain proliferation by preventing cellular senescence. Here, we report that α-catenin also drives malignant invasion and metastasis. α-Catenin was upregulated in highly invasive non-small cell lung cancer (NSCLC) cell lines, where its ectopic expression or short-hairpin RNA-mediated attenuation enhanced or limited invasion or metastasis, respectively. α-Catenin interacted with integrin-linked kinase (ILK), a serine/threonine protein kinase implicated in cancer cell proliferation, antiapoptosis, invasion, and angiogenesis. Attenuation of ILK or α-catenin reciprocally blocked cell migration and invasion induced by the other protein. Mechanistic investigations revealed that α-catenin activated Akt-NF-κB signaling downstream of ILK, which in turn led to increased expression of fibronectin and integrin ανβ3. Pharmacologic or antibody-mediated blockade of NF-κB or ανβ3 was sufficient to inhibit α-catenin–induced cell migration and invasion. Clinically, high levels of expression of α-catenin and ILK were associated with poor overall survival in patients with NSCLC. Taken together, our study shows that α-catenin plays a critical role in cancer metastasis by activating the ILK-mediated Akt-NF-κB-ανβ3 signaling axis. Cancer Res; 73(1); 428–38. ©2012 AACR.

Introduction

Lung cancer is the most common cause of cancer-related mortality (1). The high mortality rate in patients with lung cancer is due to metastatic progression of the disease (2). The process of metastasis is highly complex and requires activation of the epithelial–mesenchymal transition, remodeling of the extracellular matrix, initiation of neoangiogenesis, and migration of cancer cells to distant organs (3–5). Currently, there are no effective drugs that can inhibit cancer metastasis. Thus, understanding the molecular mechanisms that cause cancer metastasis could identify new therapeutic targets.

α-Catenin is an 82-kDa protein that shares sequence homology with human vinculin and α-catenin (6). The N-terminal region of α-catenin contains binding sites for β-catenin, talin, and α-actin. The C-terminal region of α-catenin contains potential binding sites for the tight junction protein ZO-1 and the actin cytoskeleton (6, 7). The structure of α-catenin suggests that it may function as a cytoskeletal linker protein. In addition, previous studies have found that α-catenin is involved in the Rho signaling pathway through its interaction with Lbc, a Rho guanine nucleotide exchange factor (7). The Rho family has been shown to play important roles in focal adhesion formation and in the organization of the actin cytoskeleton (8). α-Catenin may also play a role in cancer because it can directly interact with inhibitor of IκB kinase (IKK)-β and activate NF-κB signaling, which promotes cancer cell migration and resistance to apoptosis (9). We recently reported that α-catenin is upregulated in both cancer cell lines and oral squamous cell carcinomas, and knocking down α-catenin induces cellular senescence (10).

Integrin-linked kinase (ILK) is a serine/threonine protein kinase that is usually located in focal adhesions and regulates many cellular processes, including proliferation, survival, differentiation, migration, invasion, and angiogenesis (11–13). ILK can regulate the phosphorylation of Akt at Ser473 in various cell types, and this phosphorylation is required for its full activation (14). By promoting Akt phosphorylation, ILK stimulates signaling pathways that regulate cell survival and migration (15–17). Other studies have also reported that high ILK expression promotes the epithelial–mesenchymal transition and leads to invasion and metastasis by stimulating Snail expression, which in turn suppresses E-cadherin expression (18). In clinical studies, overexpression of ILK is often a prominent feature of human malignancies, and it correlates with a poor outcome (19, 20). Thus, ILK is an attractive therapeutic target in human cancers.
Although α-catulin may function as a cytoskeletal linker protein and modulate the Rho signaling pathway, its functional role in cancer metastasis is still unclear. We found that over-expressing α-catulin in cancer cells remarkably increased cancer cell metastasis both in vitro and in vivo. We elucidated the molecular mechanisms by which α-catulin regulates tumor progression using a yeast 2-hybrid genetic screen, and we identified ILK as an α-catulin–interacting protein. α-Catulin increases activation of NF-κB through an ILK-dependent pathway, which in turn increases fibronectin and integrin αvβ3 protein expression. These signaling activation events promote cancer cell migration and invasion and metastasis.

Materials and Methods

Cell culture

Four non–small cell lung cancer (NSCLC) cell lines with progressive levels of invasiveness (CL1-0, CL1-1, CL1-2, and CL1-5) were established and characterized as previously described (21). The NSCLC cell line A549 was obtained from the American Type Culture Collection and cultured in Dulbecco’s Modified Eagle Medium high glucose media supplemented with 10% FBS and antibiotics. All of the cell lines were incubated at 37°C in a humidified atmosphere with 5% CO2.

Confirmation of interaction by yeast 2 hybrid

The Saccharomyces cerevisiae strain AH109 was transformed using the yeast expression vector pAS2-1-α-catulin. Next, the transformed yeast cells were cultured in synthetic complete selective media lacking tryptophan overnight. The transformed yeast cells were transformed again with pACT2-ILK. Doubly transformed yeast cells were plated on a yeast dropout media lacking tryptophan, leucine, and histidine and supplemented with 5 mmol/L 3-amino-1,2,4-triazole and 80 μg/mL X-gal; then, they were incubated for 5 to 7 days in a 30°C incubator. Positive colonies were plated on the same synthetic complete dropout media for amplification.

Xenograft animal model

For spontaneous metastasis assay, the cells (1 × 106) were suspended in 100 μL Hank’s balanced salt solution (HBSS) and subcutaneously injected into the posterior flank of 8-week-old male nonobese diabetic–severe combined immunodeficiency (NOD-SCID) mice. The injected mice were euthanized after 12 weeks. The lungs were removed and fixed in 10% formalin. The number of lung tumor colonies was counted under a dissecting microscope. Representative lung tumors were removed, fixed, and embedded in paraffin. The embedded tissue was sectioned into 4-μm sections and the sections were stained with hematoxylin–eosin for histologic analysis.

For experimental metastasis assay, the cells (1 × 106) were suspended in 100 μL HBSS and injected into the lateral tail vein of 8-week-old SCID mice. The injected mice were euthanized after 5 weeks. The lungs were removed and fixed in 10% formalin. The number of lung tumor colonies was counted under a dissecting microscope. Representative lung tumors were removed, fixed, and embedded in paraffin. The embedded tissue was sectioned into 4-μm sections, and the sections were stained with hematoxylin–eosin for histologic analysis.

Survival analysis

The public NSCLC cohort datasets used for validation were from the University of Michigan Cancer Center (Ann Arbor, MI; ref. 22). A patient’s risk score was calculated as the sum of the levels of expression of each gene. The patients were classified as having a high-risk gene signature or a low-risk gene signature with the median-risk score serving as the threshold value. The survival curves for both groups were obtained by the Kaplan–Meier method and were compared using the log-rank test. Multivariate Cox proportional hazard regression analysis was used to evaluate independent prognostic factors associated with patient survival, with the gene signature, age, sex, and stage as the covariates. Both the log-rank test and the Cox test were 2-sided, and a P value <0.05 was considered to be statistically significant.

Results

α-Catulin enhances lung cancer cell migration and invasion

To investigate the expression level of α-catulin in several lung cancer cell lines, we used quantitative reverse transcription (qRT)-PCR and Western blotting to measure the mRNA and protein expression of α-catulin in 4 lung cancer cell lines with progressive levels of invasiveness, including CL1-0, CL1-1, CL1-2, and CL1-5. The migratory/invasive ability of the 4 human lung cancer cell lines ranges from low in CL1-0 to high in CL1-5 (21), and the levels of migration and invasiveness are positively correlated with α-catulin expression (Fig. 1A). To confirm that α-catulin can regulate cancer cell migration and invasion, the CL1-0 cells were transduced with an α-catulin expression construct in a lentivirus vector, PLKO_AS2. The CL1-5 cells were infected with 2 different lentiviruses (shB01 and shC01) that produce specific short-hairpin RNAs (shRNAs) targeting α-catulin mRNA (bases 1401–1421 and 324–344, respectively). We found that α-catulin protein expression in the transduced CL1-0 cells increased up to 4-fold (Fig. 1B, left), but the proliferation rate was similar to that of the control cells (Supplementary Fig. S1A). After 2 days of infection and puromycin selection, we found that the α-catulin protein levels were reduced by approximately 80% in the shB01- or shC01-transduced CL1-5 cells (Fig. 1B, right), and the proliferation rate of these cells was reduced by 50% (Supplementary Fig. S1B). These cells were assayed for migration and invasion using a Transwell system. The results showed that an increase of α-catulin in the CL1-0 cells promoted an approximately 3-fold increase in migration and invasion (Fig. 1C). In contrast, the migratory/invasive ability of the α-catulin–silenced CL1-5 cells was significantly decreased by more than 80% after normalization relative to the proliferation rate at the same time point (Fig. 1D). α-Catulin was also overexpressed in A549 lung cancer cells, and the results showed that α-catulin promoted the migration and invasion of the A549 cells but did not influence cell proliferation (Supplementary Fig. S1C–S1F). Taken together, these results indicate that α-catulin expression levels were positively associated with the migratory and invasive abilities of cancer cells.
α-Catulin enhances lung cancer cell metastasis in vivo

To investigate whether α-catulin can enhance lung cancer cell metastasis in vivo, we generated lung tumor metastasis models by subcutaneously injecting control, α-catulin–overexpressing CL1-0 or α-catulin knockdown CL1-5 cells into NOD-SCID mice and measured the number of lung nodule formations (Fig. 2A). After subcutaneous injection, the α-catulin–overexpressing cells induced a similar incidence of tumor formation compared with the controls with each group having 4 tumors resulting from a total of 4 inoculations. Interestingly, 12 weeks after subcutaneous injection, the mice that were injected with α-catulin–overexpressing cells displayed a significantly elevated incidence of metastatic tumors in the lung compared with the controls (Fig. 2B and C). At this time point, the mice that were injected with α-catulin–overexpressing cells had larger tumors than the mice that were injected with control cells (Supplementary Fig. S2A). In contrast, we found that the mice that were subcutaneously injected with the shC01-infected CL1-5 cells had a significantly decreased number of lung nodules compared with those injected with the shLuci-infected control cells (Fig. 2D). The incidence of tumor formation from shC01-infected CL1-5 cells was similar to the incidence in the control cells with each group having 8 tumors resulting from a total of 10 inoculations. However, the average tumor size in the mice injected with the shC01-infected CL1-5 cells was notably smaller (Supplementary Fig. S2B). We also evaluated the metastatic potential for lung colonization after tail vein injections (Fig. 2E). Five weeks after the tail vein injections, the number of lung nodule formations increased up to 4-fold in the mice injected with the α-catulin–overexpressing cells (Fig. 2F) and significantly decreased in the mice inoculated with the α-catulin–depleted cells (Fig. 2G and H). The average lung weight from the mice that were injected with the shC01-infected CL1-5 cells also decreased compared with the lungs from the control mice that were injected with the shLuci-infected CL1-5 cells (Supplementary Fig. S2C). This series of assays showed that by increasing the migratory and invasive
behavior of lung cancer cells, α-catulin promoted cancer cell metastasis in vivo.

α-Catulin enhances lung cancer cell migration and invasion through interaction with ILK

To dissect the mechanism of action of α-catulin in cancer cell migration and invasion, we carried out a yeast-2 hybrid screen using full-length α-catulin as bait to identify its interaction partners. One of the key interacting proteins that we identified was ILK (Fig. 3A). To confirm the binding of ILK to α-catulin, protein lysates were immunoprecipitated with anti-α-catulin or anti-ILK antibodies and then visualized by Western blotting. The results showed that endogenously α-catulin could interact with ILK (Fig. 3B). Next, we wanted to determine which domain of ILK interacted with α-catulin. Deletion mutants of FLAG-ILK (Fig. 3C) and full-length α-catulin-GFP were cotransfected into CL1-0 cells, and the protein lysates were immunoprecipitated with an anti-FLAG antibody and visualized by Western blotting with an anti-α-catulin antibody. As shown in Fig. 3D, the full-length and the 1–170 amino acid region of ILK interacted with α-catulin. ILK has been found to play an important role in inducing the epithelial–mesenchymal transition and in promoting cancer cell migration and invasion (18). We also found that the protein expression level of ILK ranges from low in CL1-0 to high in CL1-5 (Supplementary Fig. S3). Thus, we wanted to test whether the interaction between ILK and α-catulin mediated α-catulin–driven cancer cell migration and invasion. We used specific shRNAs to silence ILK expression in α-catulin–overexpressing CL1-0 cells (Supplementary Fig. S4A). These cells were assayed for migration and invasion using a Transwell system. The results revealed that α-catulin–overexpressing CL1-0 cells exhibited increased cell migration and invasion, and depletion of ILK expression diminished this phenomenon (Fig. 3E). Surprisingly, we found that α-catulin also plays a key role in ILK-mediated cancer cell migration and invasion. We knocked down the expression of α-catulin in ILK–overexpressing CL1-5 cells, which led to a decrease in the migratory/invasive ability induced by ILK expression (Fig. 3F, Supplementary Fig. S4B).

Figure 2. α-Catulin enhances lung cancer cell metastasis in vivo. A, a diagram of the lung metastasis experiment. Lung cancer cells were subcutaneously injected into NOD/SCID mice, and the tumor nodules were counted in the lungs of the animals. B, representative images of metastatic lung nodules (top) and histologic staining (hematoxylin and eosin) of lung sections from mice injected with control or α-catulin–overexpressing CL1-0 cells. C and D, quantitation of the metastatic lung nodules from mice injected with α-catulin–overexpressing CL1-0 or α-catulin–depleted cells. The number of lung metastatic nodules in individual mice was counted under a dissection microscope after 12 weeks. E, a diagram of the lung metastasis experiment. Lung cancer cells were injected into the tail vein of NOD-SCID mice and the tumor nodules in the lungs of the animals were counted. F and G, quantitation of metastatic lung nodules from mice injected with either α-catulin–overexpressing CL1-0 or α-catulin knockdown cells. The number of lung metastatic nodules in individual mice was counted under a dissection microscope after 35 days. H, representative images of metastatic lung nodules (top) and histologic staining hematoxylin and eosin of lung sections from mice injected with control (sh-Luci) or α-catulin–depleted CL1-5 (sh-C01) cells.
Figure 3. α-Catulin enhances lung cancer cell migration and invasion through its interaction with ILK. A, yeast 2-hybrid analysis of the interaction between ILK and α-catulin. Interactions were detected on the basis of the ability of the cotransformants to grow in the absence of histidine and exhibit β-galactosidase activity. B, protein lysates from CL1-5 cells were immunoprecipitated with IgG, α-catulin, or ILK antibodies and visualized by immunoblotting with anti-α-catulin and anti-ILK antibodies. C, a diagram of the full-length and various deletion constructs of ILK. D, coimmunoprecipitation of endogenous α-catulin and various FLAG-ILK constructs. After transfection with different FLAG-ILK constructs, immunoprecipitation was conducted with an anti-FLAG antibody. The immunoprecipitates (IP) and 10% of the whole-cell extract (WCE) were fractionated by SDS-PAGE and the immunoblots were probed with anti-α-catulin or anti-FLAG antibodies. E, knockdown of ILK reduced α-catulin–promoted cell migration and invasion. CL1-0 cells were infected with pLKO_AS2, pLKO_AS2 α-catulin, or pLKO_AS2 α-catulin combined with sh-ILK virus. The relative migratory and invasive abilities were normalized relative to those of the control cells. F, knockdown of α-catulin inhibited ILK-induced cell migration and invasion. The experiment was replicated 3 times. The data are presented as the mean ± SD of 3 independent assays.
α-Catulin–promoted activation of the Akt-NF-κB signaling pathway is ILK dependent

Previous studies have shown that Akt Ser473 could be directly phosphorylated by ILK, which results in Akt activation (14). Activation of Akt leads to IκB-α degradation and activates the NF-κB signaling pathway. Thus, we sought to elucidate whether the Akt-NF-κB signaling pathway is involved in α-catulin–regulated cancer cell migration and invasion. As shown in Fig. 4A, compared with the control cells, overexpression of α-catulin in the CL1-0 cells dramatically increased the phosphorylation of Akt at Ser473, IκB-α at Ser32, and NF-κB at Ser536. IκB is an inhibitor of NF-κB activation. The phosphorylation of IκB-α at Ser32 is required for its degradation, which in turn leads to the phosphorylation of NF-κB at Ser536 and promotes NF-κB translocation into the nucleus (23). Conversely, when we silenced α-catulin expression in the CL1-5 cells, the phosphorylation of Akt at Ser473, IκB-α at Ser32, and NF-κB at Ser536 decreased (Fig. 4B). Knockdown of ILK in the α-catulin–overexpressing cells diminished α-catulin–induced NF-κB activation (Fig. 4C). Interestingly, knockdown of α-catulin in the ILK-overexpressing cells also inhibited the activation of the Akt-NF-κB signaling pathway (Fig. 4D). Next, we wanted to test whether NF-κB activation was involved in α-catulin–induced cancer cell migration and invasion. We used a NF-κB inhibitor,

Figure 4. The interaction between α-catulin and ILK plays an important role in the AKT signaling pathway. A, CL1-0 cells were transfected with GFP or α-catulin-GFP constructs. Protein expression was assessed by Western blotting. B, CL1-5 cells were infected with lentiviruses carrying shB01 and shC01 targeting α-catulin or the negative control luciferase (shLuci) and analyzed by Western blotting. C, CL1-0 cells were transfected with GFP or α-catulin-GFP and then infected with shILKA01 and shILKB01 lentiviruses targeting ILK. The protein expression was assessed by Western blotting. D, CL1-5 cells were transfected with FLAG or ILK-FLAG plasmids and then infected with shB01 and shC01 lentiviruses. The protein expression was assessed by immunoblotting. E, pLKO_AS2 and pLKO_AS2 α-catulin–infected CL1-0 cells were treated with dimethyl sulfoxide (DMSO) or NF-κB inhibitor (BAY). The relative migratory and invasive abilities were normalized to those of the control cells. The experiment was replicated 3 times. The data are presented as the mean ± SD of 3 independent assays.
Figure 5. Integrin $\alpha_\beta_3$ is the downstream target of the $\alpha$-catulin-ILK signaling pathway, which mediates cancer cell migration and invasion. A, pathway analysis of differential global gene expression in $\alpha$-catulin-overexpressing A549 cells. MetaCore software with GeneGo Map Folders was used to analyze the pathways.
Bay 11-7082, to treat the α-catenin–overexpressing cells and found that inhibition of NF-xB effectively decreased α-catenin–induced cell migration and invasion (Fig. 4E).

α-Catenin activates an integrin-ILK-NF-κB positive feedback loop to promote cell migration and invasion

To identify the molecular mechanisms by which α-catenin affects cancer cell migration and invasion, we used a cDNA microarray to analyze the genes regulated by overexpression of α-catenin. A MetaCore software analysis of global genes revealed that cytoskeleton remodeling was the major pathway that was significantly altered in the α-catenin–overexpressing cells (Fig. 5A). Several genes involved in the reorganization of the cytoskeleton were markedly upregulated in the α-catenin–overexpressing cells (Fig 5B). In addition, many NF-xB target genes were also increased in the α-catenin–overexpressing cells, suggesting that α-catenin indeed activates the NF-xB pathway (Supplementary Table S1). The main function of ILK is to connect integrins to the cytoskeleton and to regulate cell–extracellular matrix (ECM) interactions, cytoskeletal organization, and cell signaling, with important roles in cancer metastasis (11, 24). Interestingly, we found that the ECM-integrin-ILK-GTPase signaling pathway, including fibronectin 1 (FN1), type IV collagen, integrins, CDC42, and ROCKs, is the predominant axis that is altered in α-catenin–overexpressing cells. We also checked the expression levels of α-catenin in other pair of cell lines (CL 1-1, CL 1-5F4, and PC9, PC9/IR) and found that the expression of α-catenin was positively correlated with those of ILK/FN1/TGFB3 and their invasive ability (Supplementary Fig. S5; refs. 21 and 25). Overexpression of α-catenin increased FN1 and integrin α5β1 expression, whereas knockdown of α-catenin decreased expression (Fig. 5C). FN1 is a known NF-xB target gene (26). Our results showed that ILK-Akt-NF-xB signaling is required for α-catenin–promoted cancer invasiveness. Therefore, we further examined whether α-catenin induction of FN1 and integrins is dependent on ILK-NF-xB signaling. The results showed that silencing ILK in the α-catenin–overexpressing CL1-0 cells effectively blocked α-catenin–enhanced FN1 and integrin α5β1 expression (Fig. 5D, left). In contrast, knockdown of α-catenin in the ILK-overexpressing cells diminished ILK-activated FN1 and integrin α5β1 upregulation (Fig. 5D, right). Next, we found that the α-catenin–overexpressing cells treated with the NF-xB inhibitor Bay 11-7082 had a decreased expression of FN1 and integrin α5β1 (Fig. 5E). Furthermore, we treated the α-catenin–overexpressing cells with IgG or an integrin α5β1-neutralizing antibody and found that the anti-α5β1 antibody significantly decreased cell migration and invasion (Fig. 5F). These data suggest a positive feedback loop in which, upon α-catenin overexpression, ILK-NF-xB signaling activation increases FN1 and integrin α5β1 expression, which further modulates ECM-integrin-ILK–mediated cytoskeletal reorganization to promote cell migration and invasion.

The CTNNA1 plus ILK 2-gene signature can predict survival in patients with NSCLC

The clinical characteristics of 177 patients with NSCLC from the Michigan cohort are summarized according to the risk associated with their CTNNA1 plus ILK 2-gene signature (Supplementary Table S2). The Kaplan–Meier survival curves for the high- and low-risk groups in the Michigan cohort were separable and showed a significant poor survival rate only in the subgroup exhibiting both high levels of ILK and CTNNA1 (log-rank test, $P = 0.019$; Fig. 6A–C). To show that inclusion of the 2-gene signature in the clinical model could be used to predict survival independently of the cancer stage, we conducted a multivariate Cox regression. From the initial set of 4 variables (age, gender, stage, and 2-gene signature), the final model contained only the stage and the 2-gene signature (Table 1).

Discussion

Elucidating complex oncogenic signaling pathways is important for identifying novel therapeutic targets for clinical treatment. Here, our data show that α-catenin plays a critical role in lung cancer metastasis. Expression of α-catenin was correlated with cell migratory/invasive ability in vitro and cancer metastasis in vivo in NSCLC. Moreover, we showed that α-catenin promoted the migration and invasion of NSCLC cells through the ILK/NF-xB/integrin network. α-Catenin directly interacted with ILK, which in turn activated the ILK/Akt/NF-xB signaling pathway. This led to increased expression of the NF-xB downstream genes fibronectin and integrin α5β1, which sequentially activated NF-xB signaling and resulted in cancer cell migration, invasion, and metastasis (Fig. 6D). The 2-gene signature (ILK plus CTNNA1) was even more strongly associated with clinical outcomes. Most importantly, blocking expression of α-catenin, ILK, NF-xB, and integrin α5β1 prevented cancer cell migration and invasion. The interactions between the extracellular matrix proteins, integrins, and the actin cytoskeleton are important for cell migration (27). The ILK–PINCH–parvin (IPP) complex functions as an integrin signaling adaptor. The IPP complex can act as a structural module that connects integrins to the actin cytoskeleton, and it can also act as a signaling platform that modulates various cellular processes, including cell migration (11). ILK is a central component for the assembly of the IPP containing the universal genes that were differentially expressed in α-catenin–overexpressing A549 cells. B, the expression levels of genes involved in the cytoskeleton-remodeling pathway were individually normalized to GAPDH. The data were presented as log2 ratios of the normalized expression in α-catenin–overexpressing to the expression in control cells. C, the expression of fibronectin and integrins in α-catenin–overexpressing CL1-0 and α-catenin–depleted CL1-5 cells was assessed by RT-PCR (left) and Western blotting (right). GAPDH and β-actin were used as loading controls. D, Western blotting analyzed the expression of fibronectin and integrins in α-catenin–overexpressing CL1-0 cells (left) and ILK-overexpressing CL1-5 cells (right). α-Catenin–overexpressing CL1-0 cells were infected with shILKΔ0 and shILKΔ0 lentiviruses targeting ILK. ILK-overexpressing CL1-5 cells were infected with shCATUB01 and shCATUC01 lentiviruses targeting α-catenin. E, pLKO_AS2 and pLKO_AS2 α-catenin–infected CL1-0 cells were treated with different doses of NF-xB inhibitor (BAI); RT-PCR was used to determine mRNA expression. F, pLKO_AS2 and pLKO_AS2 α-catenin–infected CL1-0 cells were treated with IgG or anti-integrin α5β1 neutralizing antibody. The relative migratory and invasive abilities were normalized to those of the control cells. The data are presented as the mean ± SD of 3 independent assays.
complex, and it contributes both its adaptor properties and its kinase activity to directly activate several signaling pathways downstream of the integrins (12). Here, we found that α-catenin interacted with multiple ankyrin (ANK) repeat domains of ILK that are required for ILK localization to focal adhesions and for ILK signaling. The interaction between α-catenin and ILK enhanced the phosphorylation of Akt at Ser473. Knockdown of α-catenin or ILK decreased Akt activation. Previous studies have shown that the ANK domain of ILK also interacts with PINCH1 (28) and the ILK-associated protein (ILKAP; ref. 29). Deletion of the ANK domain of ILK or knockdown of PINCH1 expression could disrupt ILK localization to focal adhesions and decrease Akt Ser473 phosphorylation (30). ILKAP is a type-2C protein phosphatase that binds to ILK and negatively regulates ILK signaling. These observations support the idea that ILK activity can be regulated by protein–protein interactions. In addition, α-catenin is considered to be a cytoskeletal linker protein (6) because of its structural similarity to vinculin, which links the actin cytoskeleton to integrins (31). These results suggest that α-catenin is an integrin signaling adaptor and plays a critical role in regulating integrin-mediated cellular functions via binding to ILK.

In addition to serving as the adaptor connecting integrin to the actin cytoskeleton, ILK is also a serine/threonine protein kinase. Glycogen synthase kinase 3β (GSK-3β) and Akt are the 2 major downstream substrates of ILK in cancer cells (32). ILK directly phosphorylates GSK-3β at Ser9 and induces the dissociation of GSK-3β from its substrate β-catenin, leading to the stabilization and activation of β-catenin (33). ILK also activates Akt through phosphorylation of Akt at Ser473, which regulates genes essential for survival, proliferation, and migration (16, 34). Moreover, ILK promotes melanoma, ovarian, and bladder cancer cell migration through the phosphorylation of GSK-3β and Akt (35–37). However, we found that in NSCLC cell lines, the interaction between α-catenin and ILK induced cell migration and invasion through the phosphorylation of Akt, but not GSK-3β (Fig. 3 and Supplementary Fig. S6). These data indicate that ILK might act through different downstream signaling pathways in different tissues. Consistent with this, there are several studies showing that ILK kinase function seems to be cell type–dependent. Inhibition of ILK activity in breast cancer cells resulted in a decrease in Akt phosphorylation but had no effect on nontransformed cell types, including normal breast epithelial cells and mesenchymal cells (38). In cultured sympathetic neurons, the ILK inhibitor decreased NGF-induced stimulation of GSK-3β (39).

Our data showed that the interaction of α-catenin and ILK in NSCLC cells was associated with NF-κB activation via Akt phosphorylation. Previous studies have indicated that Akt can phosphorylate IKK α/β at Ser176/178, which in turn leads to IκB-α degradation and activation of the NF-κB signaling pathway (23, 40). Moreover, α-catenin has been found to interact with IKK α/β (9). Thus, we suggest that α-catenin may link ILK/Akt with IKK α/β to promote NF-κB activation. Interestingly, we also found that α-catenin increased the expression of FN1 and integrin αβ3, which act upstream of ILK signaling. Anti-integrin αβ3-neutralizing antibody blocks α-catenin–mediated cell migration and invasion. In addition,
we found that treatment with FN1 increased NF-κB activation in CL1-0 cells, and this activation was blocked by an ILK inhibitor (Supplementary Fig. S7). It has been previously shown that α-catenin plays an important role in fibronectin-mediated wound repair and proliferation (41). Thus, after α-catenin induced upregulation of FN1 and integrin αβ3 expression via the interaction with ILK, FN1 then binds to integrins and activates the α-catenin/ILK complex in a positive feedback loop.

Currently, there are no efficient drugs that inhibit tumor progression in patients with NSCLC. Identifying the key pathways regulating tumor growth, angiogenesis, and metastasis may be helpful for developing more effective chemotherapeutics. Recent studies indicated that NF-κB is involved in cancer metastasis by regulating various cancer-related genes (42–44). Thus, the molecular mechanisms that are involved in NF-κB activation in cancer cells become more important. Our results support the hypothesis that the α-catenin/ILK complex is a key upstream regulator of NF-κB signaling. Inhibiting α-catenin, ILK, NF-κB, or integrin αβ3 prevented cancer cell migration and invasion. In addition, α-catenin is known to have oncogenic potential through its interaction with IKK-β and induction of NF-κB activity (9) and by preventing cellular senescence (10). Moreover, inhibitors of ILK (27), NF-κB (45), and integrin (46) have been used in animal model studies and clinical trials. Therefore, the α-catenin/ILK signaling pathway in NSCLC might provide new opportunities for therapeutic intervention, and the development of α-catenin inhibitors may be beneficial for cancer therapy.

We have found that silencing α-catenin in cancer cells decreased cell proliferation in vitro and in vivo (10). In this study, although α-catenin overexpression did not alter the cell proliferation rate, it promoted tumor growth in vivo (Supplementary Figs. S1A and S2A). Several studies indicate that tumor growth is not only determined by cancer cells themselves but also by the tumor microenvironment and angiogenesis (47, 48). To stimulate angiogenesis, tumors increase the production of many angiogenic factors, such as fibroblast growth factor and VEGF (49, 50). Here, we found that several angiogenic factors, including angiopeptin-1, FG2, interleukin-8, neuregulin-1, placenta growth factor, VEGFα, and VEGFC were upregulated in α-catenin-overexpressing cells (Supplementary Table S3). Taken together, α-catenin may promote tumor growth in vivo by enhancing tumor angiogenesis.

In conclusion, our data strongly suggest that the α-catenin/ILK/NF-κB/integrin αβ3 signaling pathway is associated with cell migration and invasion in NSCLC. Fibronectin and integrin αβ3 may regulate the activity of α-catenin and ILK in a positive feedback loop. Thus, this novel signaling axis may be a potential therapeutic target for treating NSCLC metastasis.

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

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Liang et al.


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