Integrin-binding Protein Nischarin Interacts with Tumor Suppressor Liver Kinase B1 (LKB1) to Regulate Cell Migration of Breast Epithelial Cells*§

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Background: This work examines the importance of interaction between Nischarin and LKB1.
Results: Nischarin and LKB1 interact in vivo. Absence of Nischarin and LKB1 increases cell migration and tumor growth.
Conclusion: This is the first report showing that two suppressors work in concert to inhibit cell migration.
Significance: Understanding the mechanisms of regulation of tumor suppressors may have many therapeutic advantages.

Biallelic inactivation of LKB1, a serine/threonine kinase, has been detected in 30% of lung adenocarcinomas, and inhibition of breast tumor growth has been demonstrated. We have identified the tumor suppressor, Nischarin, as a novel binding partner of LKB1. Our mapping analysis shows that the N terminus of Nischarin interacts with amino acids 44–436 of LKB1. Time lapse microscopy and Transwell migration data show that the absence of both Nischarin and LKB1 from an invasive breast cancer cell line (MDA-MB-231) enhances migration as measured by increased distance and speed of migrating cells. Our data suggest that this is a result of elevated PAK1 and LIMK1 phosphorylation. Moreover, the absence of Nischarin and LKB1 increased tumor growth in vivo. Consistent with this, the percentage of S phase cells was increased, as demonstrated by flow cytometry and enhanced cyclin D1. The absence of Nischarin and LKB1 also led to a dramatic increase in the formation of lung metastases. Our studies, for the first time, demonstrate functional interaction between LKB1 and Nischarin to inhibit cell migration and breast tumor progression. Mechanistically, we show that these two proteins together regulate PAK-LIMK-Cofilin and cyclin D1/CDK4 pathways.

Metastasis, a complex process involving cell growth, cell migration, invasion, and angiogenesis (1), is the leading cause of cancer-related deaths. Therefore, it is important to elucidate the molecular targets that can prevent cancer metastasis. Although tumor suppressor genes are thought to work in concert to inhibit cancer (2, 3), the molecular mechanisms in this process are not clearly understood. In 2000, we identified a novel protein, Nischarin, as an α5 binding protein (4). Nischarin is a cytosolic protein that binds preferentially to the cytoplasmic domain of the integrin α5. The Nischarin gene has been mapped to chromosome 3p21, a metastasis suppressor locus that is lost in several types of cancer (5).

Rho, Rac, and Cdc42 are the best studied members of the Rho GTPase family. Respectively, they stimulate actin stress fibers, lamellipodia, and filopodia formation and are likely to be involved in integrin-mediated control of actin organization. Nischarin inhibits Rac-induced migration and invasion in breast and colon epithelial cells (6). p21-activated kinase (PAK), a downstream effector of Rac and Cdc42, is linked to cancer. High expression of PAK is detected in several breast tumor types, and activation of PAK leads to increased DNA synthesis, cell proliferation, anchorage-independent growth (7), and tumor growth (8). Moreover, PAKs phosphorylate and activate LIM kinase (LIMK), leading to increased phosphorylation of cofilin, which is important in regulating actin turnover. Inactivation of cofilin by phosphorylation results in the polymerization of actin filaments, which leads to increased migration and invasion. Earlier, we showed that Nischarin inhibits cell migration by inhibiting PAK1 and inhibiting LIMK1 (9, 10). Importantly, we have shown that overexpression of Nischarin inhibits breast tumor growth and metastasis (11). Thus, the tumor suppressor activity of Nischarin is associated with its ability to influence the signaling of cytoskeletal regulators.

The LKB1 gene was first discovered during linkage analysis studies of Peutz-Jegher syndrome (12), a rare autosomal-dominant disorder characterized by the presence of hamartomatous polyps in the gastrointestinal tract and mucocutaneous pigmentation (13). Peutz-Jegher syndrome patients are at risk of developing cancer at multiple sites, including the lung, breast, intestine, testis, cervix, pancreas, and gastrointestinal tract (14, 15). LKB1 is a serine/threonine kinase that functions as a tumor suppressor in the small intestine (16). 30% of lung adenocarcinomas harbor a somatic LKB1 gene mutation (17). LKB1 acts as a multitasking tumor suppressor (18). It has a well established role in cell polarity (19), as well as regulation of metabolism, partly through its ability to regulate the mTOR pathway (20).

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§ The abbreviations used are: PAK, p21-activated kinase; 231, MDA-MB-231; Nisch, Nischarin; A.A., amino acid; LIMK, LIM kinase.
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Overexpression of the LKB1 protein in breast cancer cells significantly inhibited tumor growth (21). Together, these findings show that LKB1 is an important tumor suppressor.

LIP1 (LKB1-interacting protein) was identified as the first LKB1-binding protein. LIP1 is a cytoplasmic protein; its most notable feature is the presence of six tandem copies of a leucine-rich repeat motif toward the N terminus. Intriguingly, LIP1 is structurally similar to Nischarin (22). Both proteins are expressed in a punctate pattern in the cytoplasm of cells (4). Deguchi et al. (23) have recently shown that LKB1 suppresses PAK1 by phosphorylation of Thr109 in the p21-binding domain.

Based on the structural similarities between Nischarin and LIP1 and the apparent correlation of tumor suppressors LKB1 and Nischarin in regulating breast cancer, we hypothesized that there is a biochemical and functional link between these two tumor suppressors. Here we present the first evidence of that interaction. We evaluated the combined effect of Nischarin and LKB1 expression on migration, anchorage-independent growth, tumor formation, and, most importantly, metastasis of highly invasive breast cancer cells. We found that Nischarin and LKB1 cooperate to inhibit tumor cell migration. In addition, we have shown that the inhibition of cell migration is associated with down-regulation of PAK1 and LIMK1. Notably, this is the first evidence of the tumor suppressor LKB1 inhibiting LIMK1 and coflin. We also found that Nischarin and LKB1 have an enhanced effect in regulating anchorage-independent growth, tumor growth, and metastasis. Considering the importance of LKB1 and Nischarin in metastasis, these findings will be important in determining the role of the LKB1-Nischarin interaction in breast cancer and will provide a foundation for subsequent preclinical and clinical studies.

EXPERIMENTAL PROCEDURES

Coimmunoprecipitation and Western Blotting—For Nischarin-LKB1 domain binding experiments, 293T cells were transiently transfected with 5 μg each of various LKB1 deletion constructs, Myc-Nischarin deletion constructs, and full-length Myc-Nischarin or full-length LKB1 using GeneExpresso Max transfection reagent. Forty-eight hours later, the cells were lysed in FLAG lysis buffer (50 mM Tris, pH 7.4, 150 mM NaCl, 1% Triton X-100, 10% glycerol, 10 mM EDTA, and 1 mM EGTA) and protease inhibitors (2 μg/ml aprotinin, 5 μg/ml leupeptin, 1 μg/ml benzamidine, 1 mM PMSF, and 1 μg/ml pepstatin) and phosphatase inhibitors (5 mM NaF and 1 mM Na2VO4). The lysates were immunoprecipitated with appropriate antibodies and immunoblotted. Detailed information about antibodies is included in the supplemental materials.

For endogenous coimmunoprecipitation, MCF7, MCF10A, or MDA-MB-231 Nischarin cells were lysed in a modified radioimmunoprecipitation assay buffer (50 mM Tris, pH 7.4, 150 mM NaCl, 1% Nonidet P-40, 0.25% sodium deoxycholate, and 1 mM EDTA), and the lysates were immunoprecipitated overnight with appropriate antibodies or a control IgG (Sigma) at 4 °C and immunoblotted with appropriate antibodies.

Transwell Cell Migration Assays—75,000 cells were seeded onto the upper chamber of 12-well Transwell plates. Medium containing 10% FBS was placed in the lower chamber and served as a chemoattractant. Twelve hours later, the cells on the upper surface of the filter were removed by gently wiping with a cotton swab. The cells that had migrated to the Transwell were fixed and stained with crystal violet. Migrated cells were visualized by microscope. For rescue experiments, 5 μg of dominant-negative LIMK1 D460N expression vector was cotransfected with 1 μg of pRC β-Gal plasmid (Stratagene). β-Galactosidase-positive cells that migrated through the membrane during a 14-h incubation were counted by staining with 5-bromo-4-chloro-3-indolyl-β-D-galactopyranoside (X-gal).

For mitomycin C (Sigma-Aldrich) treatment, the cells were incubated with mitomycin C (10 μg/ml) for 2 h before placing on top of the Transwells.

Real Time Migration Assay—Different subsets of 231 cells were trypsinized and plated onto collagen-1-coated plates. Real time migration was performed (24). Briefly, ample space for random migration was created by scraping with a pipette tip. Phase contrast images of cells were taken at 1-h intervals for 19 h using an Olympus IX71 microscope with a 10× objective. The cells were maintained at 37 °C with 5% CO2 using a Live Cell Environmental Chamber (NEUE Group, Ontario, NY).

Cell position in sequential images was determined using slide book software, and x-y coordinates of individual cells were plotted with starting points adjusted to (0, 0). Total displacement and average speed were calculated using slide book software.

Golgi Reorientation Polarity Assays—This assay was done as described previously (25). To image Golgi positioning, the cells were fixed at 6 h postwounding and stained for Golgi and nuclei as described in the immunofluorescence method in the supplemental materials. All cells with the Golgi facing the wound front were scored positive.

Soft Agar Assay—Different subsets of 231 cells (mentioned in Table 1) were suspended in 0.3% agarose in 2× DMEM + 20% FBS at a density of 50,000 cells/well and plated on a layer of 0.5% agarose in 2× DMEM + 20% FBS (in triplicate). The soft agar assay for MCF10A (supplemental Table S1) was performed similarly with 100,000 cells with complete MCF10A media. After 6 weeks, the colonies were stained with 0.5% crystal violet, and images were taken. The colony numbers were visually calculated.

Cell Cycle Analysis—This was done by staining with propidium iodide as described (26).

Tumor Growth Assay—Animal procedures were approved by the Institutional Animal Care and Use Committee of Louisiana State University Health Science Center. Tumorigenicity assays were done as described previously (27). We implanted 100,000 MDA-MB-231 cells in 50% (v/v) Matrigel into the axillary mammary gland of 7-week-old virgin female BALB/c/nu/nu mice (n = 4). Starting at 3 days after implantation, growth of the tumors was measured with a caliper in two dimensions on a weekly basis for a total of 5 weeks and expressed as (length × width2) × 0.5. All of the experiments were repeated three times. The results were presented as the means ± S.E. After 5 weeks of transplantation, the tumors were isolated.

In Vivo Metastasis Assay—Balb c/nu/nu mice were maintained as per the guidelines of Institutional Animal Care and Use Committee of this institution, and metastasis assays were done as described (26). Briefly, 1 × 106 cells in 0.2 ml of PBS were injected into nude mice by lateral tail vein injection. Three
mice were used for each group, and the experiment was repeated three times.

**LKB1 Stable Knockdown Using Lentiviral shRNA**—Lentiviral LKB1 shRNA constructs and a scramble control construct were created in the same vector system (pLKO1) (Open Biosystems). Lentivirus stocks were prepared following the manufacturer’s protocol as done previously (28). These procedures are described in greater detail in the supplemental materials.

**H&E and Ki67 Staining**—The mice were sacrificed, and the tumors were isolated and fixed with Z-Fix solution overnight. Paraffin blocks of tumor sections were prepared, sectioned, and stained for hematoxylin and eosin and Ki67 in the Morphology and Immunology Core Facility at Louisiana State University Health Science Center.

**Statistical Analysis**—Statistical analyses were performed using PRISM 5.0 software (GraphPad, San Diego, CA). Statistical analysis for cell migration, anchorage-independent growth, Ki67 staining, and tumorigenicity was done using a nonparametric two-tailed t test.

**RESULTS**

**Nischarin and LKB1 Interact in Vivo**—Nischarin is structurally similar to LKB1-binding protein LIP1 (22). Therefore, we asked whether Nischarin could potentially interact with LKB1 in vivo. HEK 293T cells were cotransfected with Myc-Nischarin or Myc-β-gal along with FLAG-LKB1. Total lysates prepared from these cells were immunoprecipitated with anti-FLAG antibody and blotted for Nischarin using anti-Myc antibody. LKB1 immunoprecipitated Nischarin, but not β-gal (Fig. 1A). Reciprocal immunoprecipitations of Myc-Nischarin with the anti-Myc antibody also communoprecipitated FLAG-LKB1 (Fig. 1B), whereas negative control β-gal did not bind to FLAG-LKB1. These results indicate that Nischarin and LKB1 interact in vivo. This is the first report showing interaction between LKB1 and Nischarin.

**Nischarin-LKB1 Interaction Is Physiologically Relevant in Breast Cancer**—To determine whether the interaction between Nischarin and LKB1 is physiologically relevant and not due to an artifact of Nischarin and LKB1 overexpression, communoprecipitation assays were performed using MCF7 and MCF10A, which endogenously express both Nischarin and LKB1. LKB1 immunoprecipitated Nischarin (Fig. 1C); reciprocal immunoprecipitations of Nischarin also immunoprecipitated LKB1 (Fig. 1D). Moreover, we determined the interaction in an invasive cancer cell line MDA-MB-231 stably expressing Nischarin (MDA-MB-231 Nischarin cells) (11). LKB1 immunoprecipitated Nischarin in these cells as well (Fig. 1E), indicating that the interaction is physiologically relevant.

**Colocalization of Nischarin and LKB1 in the Cytosol**—To further investigate the interaction between Nischarin and LKB1, we examined their localization in MDA-MB-231 Nischarin cells (11) and in MCF7 cells by immunofluorescence microscopy. It was observed that Nischarin and LKB1 colocalize in the cytosol in MDA-MB-231 Nischarin cells (Fig. 1F) and MCF7 cells (supplemental Fig. S1), indicating that their interaction occurs in cytoplasm.

**Nischarin Enhances LKB Kinase Activity**—To determine the functional consequence of Nischarin binding on LKB1 activity, we transfected Myc-Nischarin with FLAG-LKB1 or FLAG-LKB1 with vector control in 293T cells and did a kinase assay using the FLAG immunoprecipitates. The activity of LKB1 in the absence or presence of Nischarin was evaluated by measuring transphosphorylation of myelin basic protein (29), a nonspecific exogenous substrate of LKB1 in presence of [γ-32P]ATP. LKB1 with vector control showed very low myelin basic protein phosphorylation, whereas in the presence of Nischarin, LKB1 phosphorylation of myelin basic protein was increased, indicating that Nischarin increases LKB1 activity (supplemental Fig. S2).

**Nischarin Interacts with LKB1 via Its N-terminal Region**—We mapped the region of Nischarin that interacts with LKB1. Different Myc-tagged Nischarin deletion constructs, including the N-terminal and C-terminal domains, were used for communoprecipitation assays (Fig. 2A). We found that the N terminus of Nischarin amino acids (A.A.) 1–802, but not the C terminus of Nischarin (A.A. 970–1354), interacts with LKB1 (Fig. 2B). To further narrow down the region within the N terminus Nischarin, we did interaction assays using four different deletion constructs of Nischarin (Fig. 2A), finding that only the fragment A.A. 416–624 interacted with LKB1 (Fig. 2C). This indicated that region 416–624 was critical for the interaction with LKB1.

**Nischarin Interacts with LKB1 through Its Kinase Domain**—We wished to identify the regions of LKB1 required for its association with Nischarin. Thus, we used FLAG-tagged deletion constructs of LKB1 (Fig. 3A) in communoprecipitation assays to assess the ability of these fragments to bind full-length Myc-tagged Nischarin. The region containing A.A. 44–436 interacted most strongly; other N-terminal fragments (A.A. 44–309 and 1–309) weakly interacted (Fig. 3B). The C terminus of LKB1 did not interact with Nischarin, suggesting that the interaction of LKB1 with Nischarin occurs through the N terminus of Nischarin and N-terminal kinase domain of LKB1.

**Cooperative Inhibition of Tumor Cell Migration by Nischarin and LKB1**—Our previous work shows that Nischarin inhibits cell migration in several cell types (4, 6). Others have shown that LKB1 inhibits migration in esophageal and colorectal cancer cell lines (23, 30). Because either LKB1 or Nischarin alone regulates cell migration, we hypothesized that expression of LKB1 and Nischarin together may enhance their ability to inhibit migration. To test this, we manipulated LKB1 and Nischarin expression using shRNA or ectopic expression of cDNA to generate cell lines with suppression or overexpression of each protein alone or both together (Table 1; detailed in the supplemental text) in MDA-MB-231 cells (hereafter referred to as 231). This panel of cell lines was then used to test whether LKB1 and Nischarin work cooperatively to regulate cell migration. Expression and knockdown of these proteins were confirmed by Western blotting (Fig. 4A).

We assessed the role of Nischarin and LKB1 on cell motility using time lapse microscopy of 231 cells during random migration, which enabled us to quantify migration speed and displacement. 231 cells that express both Nischarin and LKB1 (231 Nischarin) (supplemental Movie S1) exhibited a significant decrease in the speed of cell migration and displacement as compared with 231 cells expressing either Nischarin alone (231...
Nischarin + LKB1 shRNA (supplemental Movie S2) or LKB1 alone (231) (supplemental Movie S3). Conversely, the absence of both Nischarin and LKB1 significantly enhanced cell migration speed and displacement (231 LKB1 shRNA) (supplemental Movie S4 and Fig. 4, B and C).

We also analyzed the effect of Nischarin and LKB1 on cell migration using a Transwell assay. The previously used cell lines (Table 1) were allowed to migrate toward serum for 12 h. Similar to the results shown in Fig. 4 (B and C), expression of both Nischarin and LKB1 (231 Nischarin) dramatically inhibited cell migration (Fig. 4D), whereas cell lines expressing either Nischarin (231 Nisch/H11001 LKB1 shRNA) or LKB1 (231) alone were less effective than when both Nischarin and LKB1 (231 Nischarin) were present. Conversely, the absence of both Nischarin and LKB1 (231 LKB1 shRNA) led to a remarkable increase in migration. To determine whether the effect of Nischarin and LKB1 on cell migration might be caused by an indirect effect on cell proliferation, migration was measured after treatment with the proliferation inhibitor mitomycin C. We obtained the same effect on migration (supplemental Fig. S3) in the presence and absence of mitomycin C, suggesting that the effects are independent of proliferation and directly caused by...
cell migration. We also confirmed our results in the Transwell migration assay using a completely independent LKB1 siRNA oligonucleotide sequence (supplemental Fig. S4), indicating that the enhanced migration is specifically due to suppression of LKB1. It should be noted that the Transwell assay is an end point measurement of migration, whereas the random migration assay measures migration over time, providing complementary approaches to assess the effects on cell migration. Both of these approaches clearly showed that a combination of Nischarin with LKB1 is more effective in inhibiting cell migration than either protein is on its own.

**Nischarin Interacts with LKB1 to Inhibit Cell Migration**—Because the expression of both Nischarin and LKB1 cooperatively inhibits migration, and there is a physical interaction between these two proteins, we hypothesized that their interaction is important for the effects on migration. To test this, we transfected cells expressing only LKB1 (231 (LKB1 +, Nisch −)) and cells expressing neither Nischarin nor LKB1 (231 LKB1 shRNA) (LKB1 −, Nisch −) with Nischarin deletion constructs (as shown in Fig. 2), or vector control along with β-gal. Transwell migration assay was performed, and β-gal-positive cells were counted. Expression of Nischarin deletion constructs that are unable to bind LKB1 did not significantly decrease migration; however, the constructs that bind to LKB1 (416–624 Nischarin and N-terminal domain-Nisch) dramatically decreased migration. As a control, transfection of the Nischarin deletion constructs into cells without LKB1 expression (231 LKB1 shRNA) had no significant effect on cell migration (data not shown), showing the dependence on LKB1 expression. These data indicate that interaction of Nischarin with LKB1 is important to inhibit migration (Fig. 4E).

**Nischarin and LKB1 Cooperate to Inhibit Anchorage-independent Growth in MDA-MB-231 Cells**—Tumor suppressors are known to inhibit anchorage-independent growth. Our previous findings show that Nischarin inhibits anchorage-independent growth, which is inhibited by Nischarin (11). LKB1 has also been shown to inhibit anchorage-independent growth (32). Therefore, we asked whether Nischarin and LKB1 have a cooperative effect on inhibiting anchorage-independent growth. We found that 231 cells expressing both LKB1 and Nischarin had a stronger reduction in the number of colonies than did those expressing LKB1 alone (231) or Nischarin alone (231 Nischarin + LKB1 shRNA cells). Conversely, absence of both Nischarin and LKB1 (231 LKB1 shRNA cells) led to a cooperative increase in number of colonies (Fig. 4, F and G). Analyzing the effect of Nischarin and LKB1 in regulating cell migration and anchorage-independent growth in MCF10A cells yielded the same results. However, the sizes of the soft agar colonies are much smaller than those composed of 231 cells, possibly because MCF10A are nontumorigenic cells (supplemental Table S1 and supplemental Fig. S5). These results suggest that Nischarin and LKB1 synergistically function to inhibit anchorage-independent growth.

**Nischarin and LKB1 Affect Cell Polarity**—It is known that LKB1 has an important part in regulating cell polarity, which is required for proper directional migration. Thus, we hypothesized that the suppression of Nischarin and LKB1 might have an effect on cell polarity and, in turn, on cell migration. To examine the combined effect of Nischarin and LKB1 on cell polarity, we used a Golgi reorientation assay (25, 31) in which Golgi realignment is used as a marker for cell polarity. In polarized cells, Golgi realigns between the leading edge and the nucleus. Approximately 62% of cells expressing both Nischarin and LKB1 (231 Nisch cells) showed Golgi realignment. In contrast, only 23% of cells lacking Nischarin and LKB1 (231 LKB1shRNA) showed Golgi realignment, indicating that polarity is defective in these cells. In LKB1 +, Nisch − (231 cells) and LKB1 −, Nisch + cells (231 Nisch LKB1 shRNA cells), only moderate alignment was observed, with 40 and 37% of cells, respectively, in proper alignment. These data demonstrate that cells expressing both LKB1 and Nischarin have enhanced polarity, suggesting that they cooperatively regulate cell polarity. In contrast, suppression of both Nischarin and LKB1 have the least polarity. Thus, polarization defects in these cells could partially account for enhanced migration (Fig. 5).

**Combined Action of Nischarin and LKB1 on Phosphorylation of PAK1 and LIMK1**—LKB1 knockdown in 231 cells (231 LKB1 shRNA) resulted in strikingly different morphology from that in other cell lines described in Table 1. Thus, we examined the effect of LKB1 and Nischarin on the actin cytoskeleton. Expression of either LKB1 alone or Nischarin alone produces a modest decrease in stress fibers, whereas expression of both has a stronger inhibitory effect on stress fibers. These findings suggest that these proteins cooperatively regulate stress fiber formation.

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**FIGURE 1. Nischarin interacts with LKB1 in vivo.** A, Nischarin was coimmunoprecipitated with LKB1. 293T cells were cotransfected with Myc-Nischarin or Myc-β-gal along with FLAG-LKB1. The lysates were immunoprecipitated (IP) with an anti-FLAG antibody. FLAG immunoprecipitates were immunoblotted (Blot) with anti-Myc antibody (panel 1), immunoblotted with anti-FLAG–antibody (panel 2), and blotted for anti-Myc and anti-FLAG antibody (panel 3 and 4). B, LKB1 was coimmunoprecipitated with Nischarin. 293T cells were cotransfected with Myc-Nischarin or Myc-β-gal with FLAG-LKB1. The lysates were immunoprecipitated with an anti-Myc antibody and immunoblotted with an anti-FLAG–antibody (panel 1). FLAG immunoprecipitates were immunoblotted with anti-Myc antibody (panel 2). Lysates were blotted for anti-Myc and anti-FLAG antibody (panels 3 and 4). C, Nischarin was coimmunoprecipitated with LKB1 in MCF10A and MCF7 cells. The cells were lysed and immunoprecipitated with anti-rabbit-LKB1 antibody. LKB1 immunoprecipitates were probed with a monoclonal anti-Nischarin antibody (right top panel) and anti-LKB1 antibody (right bottom panel). The left panel shows expression of Nischarin (top) and LKB1 (bottom) in MCF7 and MCF10A cells. The lysates were blotted for anti-Nischarin and anti-LKB1 antibody. Mouse IgG was used as a control. D, LKB1 was coimmunoprecipitated with Nischarin in MCF10A and MCF7 cells, which were lysed and immunoprecipitated with anti-mouse Nischarin. The Nischarin immunoprecipitates were probed with a monoclonal anti-Nischarin antibody (bottom panels) and anti-LKB1 antibody (top panels). Mouse IgG was used as a control. The corresponding secondary HRP-conjugated true blot antibody was used as described under “Experimental Procedures.” E, interaction of endogenous LKB1 and Nischarin in MDA-MB-231 Nischarin cells. The cells were lysed and immunoprecipitated with rabbit-anti-LKB1 antibody. Mouse IgG was used as a control. The immunoprecipitates were probed with mouse anti-Nischarin (top panel) antibody and mouse-anti-LKB1 antibody (bottom panel). Corresponding secondary HRP-conjugated true blot antibody was used as described under “Experimental Procedures.” F, Nischarin localizes with LKB1 in the cytoplasm. MDA-MB-231 Nischarin cells were plated on fibronectin-coated cover slips overnight and stained with mouse anti-Nischarin and rabbit anti-LKB1 antibodies. The cells were observed under a confocal microscope with a 60× oil immersion objective. MDA-MB-231 Nischarin cells show anti-Nischarin in green and anti-LKB1 staining in red; an overlay image is shown in which yellow represents localization of Nischarin and LKB1 in the cytoplasm. Scale bar, 50 μm.
LKB1 knockdown MDA-MB-231 cells showed more stress fibers than did other cells (Fig. 6A), leading us to hypothesize that Nischarin and LKB1 affect migration through actin-regulated proteins.

PAK1 phosphorylates LIMK1 at threonine 508 and activates LIMK1; this in turn phosphorylates cofilin, leading to inactivation of F-actin depolymerization and resulting in accumulation of actin filaments (33). LKB1 inhibits PAK1 activation (23). We have shown that Nischarin inhibits PAK1 and LIMK1 activity to inhibit migration and invasion (9, 10). However, the effect of LKB1 on LIMK activation is not known. Therefore, we investigated whether Nischarin and LKB1 have cooperative effects on the PAK1, LIMK1, and cofilin signaling cascade, which is important in regulation of the actin cytoskeleton and cell migration. The presence of either Nischarin (231 Nischarin + LKB1 shRNA cells) or LKB1 (231 cells) alone was insufficient to inhibit activation of PAK1, LIMK1, and cofilin. In contrast, when both Nischarin and LKB1 were present (231 Nischarin), the phosphorylation of PAK1, LIMK1, and cofilin was decreased. An absence of both Nischarin and LKB1 (231 LKB1 shRNA) resulted in enhancement of phosphorylation of PAK1, LIMK1, and cofilin (Fig. 6B and supplemental Fig. S6). These findings
clearly indicate that Nischarin and LKB1 have a cooperative effect in down-regulating the phosphorylation of PAK1, LIMK1, and cofilin.

Suppression of LIMK-1 Activation Decreases Migration—To confirm that an increase in LIMK1 activity is required for the increase in migration because of the absence of Nischarin and LKB1, we transfected (231 LKB1 shRNA) cells with dominant-negative LIMK1 (D460N) or vector control along with FLAG-LKB1 and did Transwell migration assay. Expression of dominant-negative LIMK decreased migration (Fig. 6, C and D), indicating that LIMK activity is required to increase migration in cells lacking both Nischarin and LKB1.

LKB1 and Nischarin Cooperatively Inhibit Tumorigenesis in a Nude Mouse Model—To confirm the in vitro effect of Nischarin and LKB1 observed in a soft agar assay, different subsets of 231 cell lines used previously were tested for tumorigenic ability in nude mice. Tumor growth was monitored after implanting these cell lines into the auxiliary mammary fat pads of BALB/c nu/nu mice. The size of tumors was determined by

| TABLE 1 |
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| **Expression of Nischarin and LKB1 in various MDA-MB-231 cell lines** |

| Cell lines             | LKB1     | Nischarin |
|------------------------|----------|-----------|
| MDA-MB-231 + scrambled shRNA | +         | –         |
| MDA-MB-231 Nischarin + scrambled shRNA | +         | +         |
| MDA-MB-231 + LKB1 shRNA   | –         | –         |
| MDA-MB-231 Nischarin + LKB1 shRNA | –         | –         |


FIGURE 3. Identification of regions of LKB1 that are crucial for interaction with Nischarin. A, a schematic of different LKB1 deletion constructs used to determine the critical region of LKB1 for interaction with Nischarin. CRD, C terminus regulatory domain. B, Nischarin interacts with the region encompassing the kinase domain of LKB1. 293T cells were cotransfected with FLAG-LKB1 and Myc Nischarin or Myc-β-gal, FLAG-LKB1 (A.A. 1–309) with Myc-Nischarin, FLAG-LKB1 (A.A. 44–436) with Myc-Nischarin, FLAG-LKB1 (A.A. 44–436) with Myc-Nischarin, and FLAG-LKB1 (A.A. 44–436) with Myc-Nischarin. The lysates were immunoprecipitated with anti-Myc antibody and immunoblotted with anti-FLAG antibody (upper right panel) and anti-Myc antibody (lower right panel). The lysates were also immunoblotted with anti-Myc antibody (lower left panel) and anti-FLAG antibody (upper left panel).
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FIGURE 4. Effect of Nischarin and LKB1 on cell migration and anchorage-independent growth. A, LKB1 expression was depleted in 231 and 231 Nisch cells. Stable pools of LKB1-depleted cells were analyzed for LKB1 expression by immunoblotting with LKB1 antibody in different 231 cells (as shown in Table 1), and vinculin was used as a loading control. The cells were also immunoblotted for Nischarin. scr shRNA, scrambled shRNA. B, analysis of displacement and speed by random migration assay. Various subsets of 231 cells (as shown in Table 1) were monitored by time lapse microscopy for 19 h. Displacement and speed of migration were quantified as described under “Experimental Procedures.” Box and whisker plots representing the total displacement and speed were generated from each group. The data represent at least 18 cells pooled from three independent experiments. Statistical analysis comparing the total displacement and average speed was done using a Mann-Whitney test. Pairwise statistical analysis was done for different cell lines. The value is provided in the graph. C, migration tracks of 231, 231 Nisch, 231 LKB1 shRNA, and 231 Nisch + LKB1 shRNA. Tracks from 10 random cells were plotted using Microsoft Excel. D, absence of Nischarin and LKB1 increases migration of 231 cells. Transwell cell migration assays were done as described under “Experimental Procedures.” Various subsets of 231 cells (as shown in Table 1) were plated on Transwells; migrating cells were counted after staining with crystal-violet. E, Nisch binds with LKB1 to regulate cell migration. MDA-MB-231 control cells (LKB1+, Nisch−) were transiently transfected with different deletion constructs of Nischarin as described in the legend to Fig. 2 plus Myc-β-gal or vector control plus Myc-β-gal. After 24 h of transfection, in vitro Transwell migration assay was done, and β-gal-positive cells were counted. F, a representative photograph showing soft agar colonies after 6 weeks of plating. Colonies were counted from different subsets of 231 as shown in Table 1. The number of soft agar colonies presented is the average of three different experiments. G, quantitative data of anchorage-independent growth assay: 231 (LKB1+, Nisch−), 231 Nisch (LKB1+, Nisch+), 231 LKB1 shRNA (LKB1−, Nisch−), and 231 Nisch + LKB1 shRNA (LKB1−, Nisch+) cell lines were plated on agar. The total number of colonies was counted after 6 weeks. The results shown are the averages of three independent experiments.

FIGURE 5. Cells lacking Nischarin and LKB1 show defective cell polarity. Different subsets of MDA-MB-231 cells: 231 (LKB1+, Nisch−), 231 Nisch (LKB1+, Nisch+), 231 LKB1 shRNA (LKB1−, Nisch−), and 231 Nisch + LKB1 shRNA (LKB1−, Nisch+), were plated on collagen-coated plates. A wound was created in tissue culture plate with cells; cells were fixed and stained for Golgi with GM 130 and nucleus with Hoechst for 6 h. A, representative confocal images of cells stained for Golgi and nucleus after 6 h of migration. Red carets represent examples of polarized cells: Golgi (green) and nucleus (blue). The white solid lines represent the wound. B, graph showing quantitation of polarized cells at 6 h after wounding.

effects of Nischarin and LKB1 from 231 cells produced larger tumors than those originating from cells expressing both LKB1 and Nischarin (Fig. 7C). We assessed cell proliferation by immunohistochemical staining of Ki67 in sections from xenograft tumors and quantified the number of Ki67-positive cells. An antiproliferative effect was seen when both LKB1 and Nischarin were present in tumor sections, whereas enhanced proliferation was noted in tumor sections when Nischarin and LKB1 expression was reduced (Fig. 7, D and E). Moreover, PAK1, which increases proliferation and tumor growth (34), is
FIGURE 6. Nischarin and LKB1 regulate migration (via actin reorganization). A, effect of Nischarin and LKB1 on number of stress fibers. Cells were fixed and stained with Alexa Fluor 594-conjugated phalloidin for 1 h. Average integrated intensity was calculated for the cell lines using Fluoview 1000 software (right panel). Original magnification, 40×. Scale bar, 40 μm. B, effect of Nischarin and LKB1 on phosphorylation of PAK1, LIMK1, and cofilin. 231 (LKB1+/Nisch), 231 Nisch (LKB1+/Nisch−), 231 LKB1 shRNA (LKB1+/Nisch−), and 231 Nisch/LKB1 shRNA (LKB1+/Nisch−) cells were lysed and immunoblotted with phospho-PAK1, phospho-LIMK1, and phospho-cofilin, total PAK1, LIMK1, and cofilin. C, LIMK1 suppression leads to inhibition of migration in cells lacking Nischarin and LKB1. 231 LKB1 shRNA cells were transiently transfected with LIMK1 D460N plus Myc-β-gal, vector control plus Myc-β-gal. After 24 h of transfection, an in vitro Transwell migration assay was done, and β-gal-positive cells were counted. D, Western blot showing the expression of phospho-LIMK1 in 231 + LKB1 shRNA transfected with LIMK1 D460N or its control.
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A

B

C

D

E

F

G

[Images and graphs related to the interaction of Nischarin and LKB1 in breast cancer, including tumor volume, tumor weight, histological images, and protein expression analysis.]
associated with cyclin D1 expression (35). Thus, we assessed cell proliferation by FACs, using propidium iodide staining of synchronously growing cells. After 48 h, the presence of both Nischarin and LKB1 produced a reduced number of cells in the S phase. In contrast, absence of Nischarin and LKB1 (231 LKB1 shRNA) significantly increased the number of cells in the S phase (Fig. 7F). In corroboration of these findings, absence of Nischarin and LKB1 from 231 cells (231 LKB1 shRNA cells) yielded a significant increase in cyclin D1 and CDK4 expression (Fig. 7G), whereas the presence of Nischarin and LKB1 (231 Nischarin) resulted in a synergistic decrease in cyclin D1 and CDK4 expression. Taken together, these findings strongly suggest that Nischarin and LKB1 cooperate in regulating tumor growth through the cyclin D1/CDK4 pathway.

**LKB1 and Nischarin Regulate Lung Metastasis**—To assess the combined effect of Nischarin and LKB1 in regulating the metastatic potential of 231 cells (231 LKB1 shRNA cells), we used an experimental metastasis model. These cells were injected into the tail veins of 4- to 6-week-old BALB/c/nu/nu mice; lung metastases was analyzed after 30 days. Consistent with our migration results, the presence of Nischarin and LKB1 together (231 Nischarin) strikingly reduced metastases, which were dramatically increased in the absence of both Nischarin and LKB1. We found that 231 cells expressing LKB1 alone (231 cells) or Nischarin alone (231 Nischarin + LKB1 shRNA cells) had fewer metastases than did stable 231 cells having no expression of Nischarin and LKB1 (231 LKB1 shRNA cells) (Fig. 8A).

Hematoxylin and eosin staining data (Fig. 8B) showed that lungs were heavily infiltrated with metastases when cells lacking Nischarin and LKB1 (231 LKB1 shRNA) were injected. As expected, few metastases were formed in lungs injected with cells expressing Nischarin and LKB1 (231 Nischarin cells). The presence of either Nischarin (231 Nischarin LKB1 shRNA) or LKB1 (231 cells) alone was insufficient to completely suppress the metastatic activity of MDA-MB-231 cells, further emphasizing the importance of cooperation between Nischarin and LKB1 in regulating metastasis.

Our previous results indicated that Nischarin expression is lower in breast tumor tissues than in normal tissues (11). To assess the expression of LKB1 in breast tumor tissue, we did quantitative PCR on 21 pairs of matched breast tissues. We observed that LKB1 expression is also low in tumor tissues as compared with matched normal tissues (supplemental Fig. S7).

**DISCUSSION**

Tumor cell migration and invasion are important in tumor growth and metastasis (36). We have shown a biochemical connection between two tumor suppressors, Nischarin and LKB1, demonstrating that Nischarin is a novel binding partner of LKB1. We found that LKB1 interacted with the N terminus of Nischarin and, more specifically, that the amino acids 416-624 of Nischarin are crucial for this interaction. This region encompasses the integrin-binding domain, which was initially used as a bait to identify Nischarin (4). The N terminus of Nischarin binds to the C-terminal kinase domain of PAK1 (9) and to the PDZ and kinase domain of LIMK1 (25), suggesting that Nischarin may function as a scaffold protein. Our previous findings indicate that Nischarin suppresses tumor cell migration, invasion, and tumor growth through the PAK-LIMK signaling cascade. LKB1 tumor suppressive function is regulated mainly through two different mechanisms: translocation of LKB1 from the nucleus to the cytoplasm and kinase activation. Interestingly, our results indicate that Nischarin enhances LKB1 kinase activation independent of STRAD and MO-25, which provides an additional mechanism of regulation of LKB1 activity.

LKB1 has been shown to colocalize with Cdc42 and PAK at the leading edge of cells, which leads to increased PAK phosphorylation (25). LKB1 interacts with active Cdc42 and active PAK, thus enhancing downstream cell polarity events (25). In contrast, LKB1 inhibits PAK1 activity. Thus, these effects might depend on cell type. LKB1 knockdown of human colorectal cells causes an increase in migration and PAK1 activity (23). Loss of epithelial cell polarity has been associated with increased cell invasion (37), and loss of LKB1 has been shown to result in aberrant lung cancer polarity (25), indicating that LKB1 is important in invasion and metastasis through its regulation of cell polarity. Our data suggest that suppression of LKB1 and Nischarin enhances cell migration and tumor growth, probably because of defective cell polarity. This is interesting, but the effects of Nischarin and LKB1 on cell polarity need to be explored in greater detail.

We have assessed the combined effect of Nischarin and LKB1 in migration by two methods: a live cell microscopy assay to track migration over time and a Transwell migration end point assay. We have found enhanced inhibitory effect in presence of both Nischarin and LKB1; however, in the absence of either of them, the effect was reduced as opposed to not having the inhibitory effect at all. These data suggest some of the effects that we observed might be independent of Nischarin-LKB1 interaction.

We demonstrated that there is an increase in phosphorylation of PAK1 in the absence of both of these proteins. Also, we found that phosphorylation of the downstream target of PAK1, LIMK1, is elevated on suppression of both LKB1 and Nischarin. LIMK1, a cytoskeletal protein, regulates the actin cytoskeleton through cofilin, an actin depolymerization protein (33). LIMK1...
is activated by several proteins, including Rho, Rac, CDC42, and PAK (38). LIMK1 is highly expressed in invasive breast cancer cell lines and invasive tumors (7), suggesting a pro-tumorigenic function of LIMK. In support of this, the LIMK pathway has been shown to have a major role in cell migration and invasion (8), as well as tumor growth and metastasis (7). This study, for the first time, shows that LKB1 inhibits LIMK phosphorylation to regulate cofilin functions.

LKB1 gene inactivation has been demonstrated in subsets of lung and pancreatic cancer, and the loss of LKB1 expression seems to be a common event in patients with higher grade invasive breast carcinoma and ductal carcinoma in situ (39). LKB1 gene mutations have been detected in Peutz-Jegher syndrome patients, who are at high risk for several cancers, including breast cancer. Although no mutations have yet been identified in breast cancer, loss of heterozygosity associated with chromosome 19p has been reported (40). In addition, low LKB1 protein levels correlate with poor prognosis in breast carcinoma (41). Consistent with this finding, we found that LKB1 expression is lower in breast cancer tissues than in normal tissues. The A.A. 44–436 region of LKB1 showed strong interaction with Nischarin. Nischarin and LKB1 localized in the cytosol of breast cancer cells. This is consistent with the observation that LKB1 lacking a nuclear localization signal enhances the cytoplasmic pool of LKB1, which is important in mediating its tumor suppressor functions (42).

Conditional deletion of LKB1 in mammary glands induces grade 2 invasive ductal carcinoma tumors after 45–85 weeks (43). Our studies support the concept that LKB1 is important in breast cancer suppression and that inhibition of LKB1 expression increases tumor cell migration, anchorage-independent growth, tumor growth, and metastasis. Several reports have indicated that some tumor suppressors function in concert to inhibit tumor growth (3). Deng et al. (3) demonstrated that Fus1 and p53 exhibit synergistic tumor suppression of lung cancer, which is due to down-regulation of double minut-2 expression (3). However, no reports have shown how tumor suppressors work in concert to inhibit breast cancer. Two apoptosis gene family members, XIAP and survivin, cooperate and interact to promote tumor invasion and metastasis (44). Also, a cancer-amplified transcriptional coactivator (ASC-2) and E2F-1 synergistically activate genes required for cell proliferation (45). Although it has been shown that LKB1 synergizes with PTEN to inhibit bladder cancer (2), there is no evidence to show cooperation of LKB1 with another tumor suppressor in breast cancer. To determine a functional link between Nischarin and LKB1, we have modulated their expression so that both are expressed, both are absent, or only one is expressed. We have shown that in a nude mouse model, the absence of both LKB1 and Nischarin has an enhanced effect on anchorage-independent cell growth, tumorigenesis, and metastasis. Here, we show for the first time that there is a functional and biochemical link between the tumor suppressors LKB1 and Nischarin in breast cancer.

Nischarin inhibits cyclin D1 expression3 and LKB1 regulates cyclin D1 expression (46), suggesting that the two may have
similar functions. Consistent with this idea, the absence of Nischarin and LKB1 enhances cyclin D1 and CDK4 expression, possibly causing increased tumor growth. PAK1 is required for the growth of breast and NSCLC tumors; targeting of PAK1 induces apoptosis of tumor cells (34). Therefore, several possibilities exist whereby Nischarin and LKB1 proteins work together to inhibit tumor growth, either through their combined effect on cell cycle arrest, driven by inactivation of PAK1, and/or through the effect on actin-regulated proteins such as PAK1, LIMK1, and cofilin. In conclusion, Nischarin and LKB1 exert cooperativity in down-regulating PAK1, leading to down-regulation of cell-cycle associated proteins and phosphorylation of proteins involved in migration and invasion (Fig. 8C). These changes ultimately affect tumor growth and metastasis. However, it is possible that other mechanisms of LKB1 and Nischarin that are not discussed in the current study, may also regulate cell migration and tumorigenesis.

In summary, we have demonstrated that Nischarin and LKB1 regulate migration, anchorage-independent growth, tumor growth, and metastasis. Also, we have defined a novel pathway of PAK1-LIMK1-cofilin through which LKB1 exerts its function of suppressing tumor cell migration. The present study could be important in unraveling the mystery of the multiple functions of LKB1.

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