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ORIGINAL ARTICLE

Regulation of triple-negative breast cancer cell metastasis by the tumor-suppressor liver kinase B1

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Liver kinase B1 (LKB1), also known as serine/threonine kinase 11 (STK11), has been identified as a tumor suppressor in many cancers including breast. Low LKB1 expression has been associated with poor prognosis of breast cancer patients, and we report here a significant association between loss of LKB1 expression and reduced patient survival specifically in the basal subtype of breast cancer. Owing to the aggressive nature of the basal subtype as evidenced by high incidences of metastasis, the purpose of this study was to determine if LKB1 expression could regulate the invasive and metastatic properties of this specific breast cancer subtype. Induction of LKB1 expression in basal-like breast cancer (BLBC)/triple-negative breast cancer cell lines, MDA-MB-231 and BT-549, inhibited invasiveness *in vitro* and lung metastatic burden in an orthotopic xenograft model. Further analysis of BLBC cells overexpressing LKB1 by unbiased whole transcriptomics (RNA-sequencing) revealed striking regulation of metastasis-associated pathways, including cell adhesion, extracellular matrix remodeling, and epithelial-to-mesenchymal transition (EMT). In addition, LKB1 overexpression inhibited EMT-associated genes (CDH2, Vimentin, Twist) and induced the epithelial cell marker CDH1, indicating reversal of the EMT phenotype in the MDA-MB-231 cells. We further demonstrated marked inhibition of matrix metalloproteinase 1 expression and activity via regulation of c-Jun through inhibition of p38 signaling in LKB1-expressing cells. Taken together, these data support future development of LKB1 inducing therapeutics for the suppression of invasion and metastasis of BLBC.

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INTRODUCTION

Distinguishing breast cancer subtypes, such as luminal and basal subtypes, at the molecular level has revealed unique signaling, transcriptome and epigenome signatures for each that defines their phenotype.[1–3] In the case of clinically diagnosed 'triple-negative breast cancer', a lack of estrogen receptor (ER), progesterone receptor (PR) and Her2/Neu overexpression, precludes the use of therapeutic agents that target these receptors.[4] In general, the basal subtype, which shares features with the triple-negative breast cancer cells,[5] is among the most lethal breast cancer subtype, characterized by a highly aggressive and metastatic phenotype.[4,6] Although pathways that may represent targets for novel therapeutic intervention for these subtypes have begun to be elucidated,[7–9] the ability to define and selectively target the invasive and metastatic phenotype of basal-type/triple-negative breast cancer remains a critical need and a major challenge facing the breast cancer field.[5]

The liver kinase B1 (LKB1/STK11 (serine/threonine kinase 11)) signaling pathway has an established role in defining cellular responses to changes in energy homeostasis and metabolism, cell polarity and stress responses.[10] The tumor-suppressor activity of LKB1 was initially identified by genomic loss in Peutz–Jeghers syndrome patients, who display an increased lifetime risk of developing various cancers, including breast.[11,12] In addition, decreased expression of LKB1 has been demonstrated in breast as well as somatic mutation or loss-of-function in a number of other solid tumor cancers.[11–14] A better understanding of LKB1 mechanisms in lung, melanoma and gastric cancers has emerged, but the role of LKB1 expression and signaling in basal subtype remains to be clearly defined.

In this study, we examine the effects of LKB1 expression in basal breast cancer cells based on the strong correlation between LKB1 expression and survival observed specifically in basal-like breast cancer (BLBC) patients by Kaplan–Meier database analysis. Through the molecular studies presented here, we define a role for LKB1 signaling in the suppression of metastasis in the BLBC subtype. Furthermore, our findings reveal LKB1 regulation of the p38(MAPK14)/mitogen-activated protein kinase (MAPK)/AP-1 signaling pathway for the regulation of matrix metalloproteinase 1 (*MMP1*). Our data identify a unique function of LKB1 in the suppression of the invasive and metastatic properties characteristic of the basal subtype of breast cancer, while upholding the tumor-suppressive role of the LKB1 pathway. Our findings further support the development of novel therapeutics to induce the LKB1 pathway in this lethal breast cancer subtype.

RESULTS

LKB1 expression is positively correlated with patient survival in basal subtype breast cancer

Although LKB1 has been shown to be a tumor suppressor in breast cancer, with reduced expression linked to decreased

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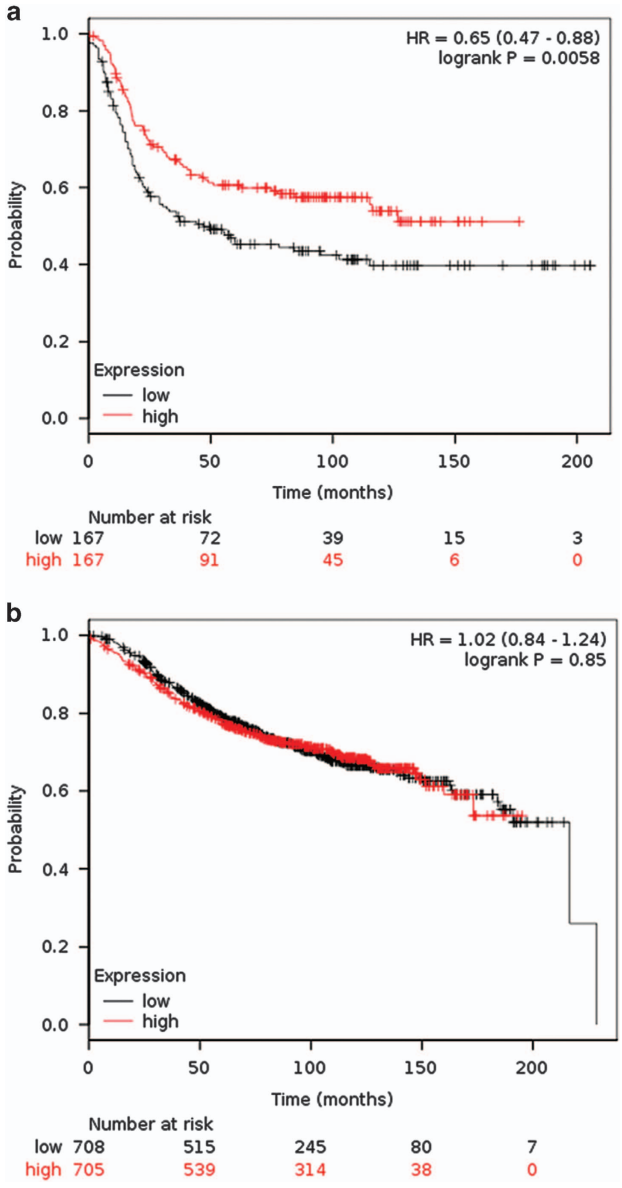

Figure 1. LKB1 expression is associated with increased survival of basal breast cancer patients. Kaplan–Meier analysis was conducted, using the Breast Cancer Kaplan–Meier Plotter,[16] for LKB1 gene expression in patients with (**a**) basal subtype ($n = 383$) or (**b**) ER-positive ($n = 1440$) breast cancer. HR indicates relapse-free survival. Red line represents high gene expression, whereas the black line represents low gene expression.

disease-free and overall survival,[15] its role in subtype selective patient survival has not been examined. Available databases for gene expression profiles in breast cancer patients [16] were used to determine if altered expression of LKB1 was associated with recurrence-free survival (RFS) in a cohort of breast cancer patients (Figure 1). Comparison of LKB1 gene expression in 383 patients with basal breast cancer (Figure 1a) to 1440 patients with ER+ breast cancer (Figure 1b) revealed a strong association of increased LKB1 expression with an overall increase in patient RFS in basal subtype (hazard ratio (HR) = 0.60, $P < 0.005$), but was not associated with RFS in ER+ cohort (HR = 1.02, $P = 0.86$).

Selective suppression of breast cancer cell invasion by LKB1

Based on the strong association between LKB1 expression and survival specifically in patients with basal subtype breast cancer, we investigated the biological impact and mechanisms of LKB1 signaling in BLBC cells. Western blot analysis revealed decreased expression of both total and phospho-LKB1 protein levels in the basal B subtype breast cancer cell lines MDA-MB-231, BT-549 and MDA-MB-157 cells compared with the ER+ MCF-7 and MDA-MB-361 cells (Figure 2a). To interrogate the role of LKB1 in these basal subtype breast cancer systems, we generated wild-type LKB1 (-LKB1) or empty vector (-VEC) cells in the MDA-MB-231 and BT-549 cell lines. Following transfection and marker selection, cell populations were pooled. Total expression and phosphorylation status of LKB1 was confirmed by western blot analysis (Figure 2b).

In addition to its role in energy homeostasis, metabolism, stress responses and tumorigenesis, LKB1 expression and activity has shown to regulate cell polarity and metastasis in other invasive cancers including lung and melanoma.[17–19] We examined the effects of LKB1 expression on the migratory and invasive abilities of MDA-MB-231 and BT-549 cells because of the highly invasive and metastatic nature of the basal subtype. Using Boyden transwell assays, we observed significant suppression of *in vitro* invasive activity in LKB1 stable expressing cells compared with vector (Figure 2c). LKB1 expression significantly inhibited the migration of BT-549 cells though to a lesser extent, whereas the migration of MDA-MB-231 cells remained unaffected (Figure 2d). No effect on proliferation was observed (Supplementary Figure 1).

LKB1 suppresses basal breast cancer cell tumorigenesis and metastasis

To determine if the anti-invasive effects of LKB1 observed *in vitro* translated to the *in vivo* setting, an immunocompromised mouse xenograft model was used as previously described[20,21] (Figure 3). MDA-MB-231-vector or -LKB1 cells were injected orthotopically into the mammary fat pad of female SCID/beige mice and tumor volume recorded twice weekly. Primary tumor volume of LKB1-expressing cells was reduced ($P < 0.05$) compared with vector cells (Figure 3a). Furthermore, a profound reduction ($P < 0.01$) in lung metastasis was observed (Figure 3b), with $>95\%$ less metastatic tumor volume within the lungs of animals injected with LKB1-expressing cells compared with vector (Figure 3c). These results show that in addition to an effect on primary tumorigenesis, LKB1 exerts significant repression on metastasis from the primary orthotopic sites.

Whole-transcriptome analysis reveals LKB1-mediated regulation of cell invasion and metastasis-associated genes

To further elucidate the mechanism of the anti-invasive and anti-metastatic effects of LKB1 in the basal subtype, duplicate samples of total RNA from MDA-MB-231-vector and MDA-MB-231-LKB1 cells were analyzed for whole-transcriptome changes by RNA-seq. Gene expression analysis revealed that the expression levels of 1579 genes were significantly ($P < 0.05$) altered following LKB1 overexpression, with 616 genes upregulated and 763 genes downregulated >0.5-fold (Figure 4a). GeneGo pathway analysis revealed significant association of downregulated genes with signaling pathways involved in extracellular matrix (ECM) remodeling, epithelial-to-mesenchymal transition (EMT) and cell adhesion, all known mediators of metastasis (Figure 4b).

Expression changes in a subset of genes associated with EMT and metastasis were independently confirmed by quantitative real-time PCR in both the MDA-MB-231 and the BT-549 cell lines (Figure 4c). With the exception of CDH1, all genes tested revealed significant expression changes in both cell lines at or beyond the levels observed in the RNA-seq data, further verifying our results. Although no significant change in CDH1 expression was observed in the BT-549 cell line, a significant increase in both gene expression (Figure 4c) and protein levels (by enzyme-linked immunosorbent assay, Supplementary Figure 2) was observed in

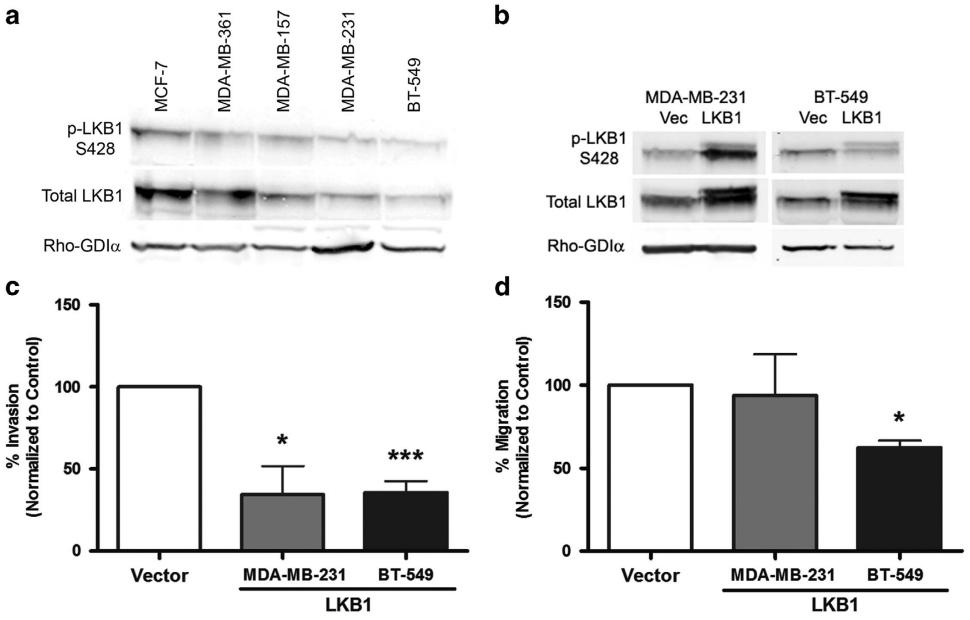

Figure 2. LKB1 expression is associated with a less invasive phenotype in breast cancer cells. (**a**) Western blot for basal levels of LKB1 (phospho-S428, and total) protein expression across breast cancer cell lines. Rho-GDIα served as a loading control. (**b**) Western blot confirmation of LKB1 (phospho-S428, and total) protein expression in pooled populations of MDA-MB-231 and BT-549 cells transfected with wild-type LKB1 or vector control. Rho-GDIα served as a loading control. (**c**, **d**) Boyden transwell (**c**) invasion and (**d**) migration assays of LKB1-overexpressing cell lines. (**c**) In all, 2.5 × 104 cells or (**d**) 5×10^4 cells were seeded in the upper chamber of a transwell insert (8 μM pore size) alone (migration) or coated with Matrigel (invasion). Lower wells contained DMEM supplemented with 10% fetal bovine serum (FBS). After 24 h, migrated or invaded cells were fixed and stained for visualization. Bars represent normalized percent migration or invasion compared with the cell line-specific vector control (set to 100%) ± s.e.m. Experiments were conducted in triplicate. *$P < 0.05$; ***$P < 0.001$.

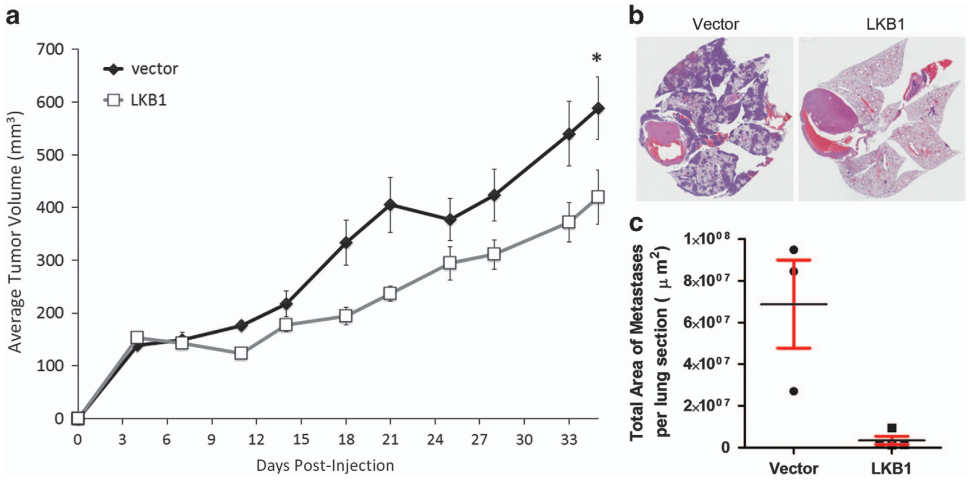

Figure 3. Expression of LKB1 inhibits MDA-MB-231 tumorigenesis and metastasis *in vivo*. Four- to 6-week-old female SCID/beige mice were injected bilaterally in the mammary fat pad with 5×10^6 MDA-MB-231-vector or -LKB1 cells suspended in 50 μl of sterile phosphate-buffered saline mixed with 100 μl reduced growth factor Matrigel; $n = 5$ mice per group. (**a**) Tumors were measured twice weekly with digital caliper. Data points represent average tumor volume (mm^3) ± s.e.m. (**b**, **c**) At day 34 post cell injection, primary tumors were removed and animals allowed to continue to monitor for distant metastasis. Two weeks later, animals were killed and lungs and livers harvested and fixed in formalin. (**b**) Hematoxylin and eosin (H&E) staining of representative lungs from each group demonstrating decreased metastasis in the LKB1 overexpression injected animals. (**c**) Quantitation of metastasis. Points represent total area of metastases per lung section ± s.e.m. Vector, $n = 3$; LKB1, $n = 4$. *$P < 0.05$.

the MDA-MB-231-LKB1 cells, consistent with the RNA-seq data. MMP-1 expression was vastly suppressed by LKB1 expression, with a >70-fold reduction in expression in the MDA-MB-231-LKB1 cells and nearly sixfold reduction in the BT-549-LKB1 cells ($P < 0.001$).

A transcription factor reporter array was used to screen for altered activity of 45 transcription factors by LKB1 in the MDA-MB-231 cells, as EMT and ECM remodeling pathways can be regulated by a number of transcription factors, including AP-1, SP-1 and NFκB. AP-1 was identified as the top downregulated transcription factor in the MDA-MB-231-LKB1 cells compared with vector (Supplementary Figure 3, Supplementary Table 1). These data further validate our RNA-seq findings of decreased expression of many genes associated with AP-1 signaling, most notably the MMP-1.

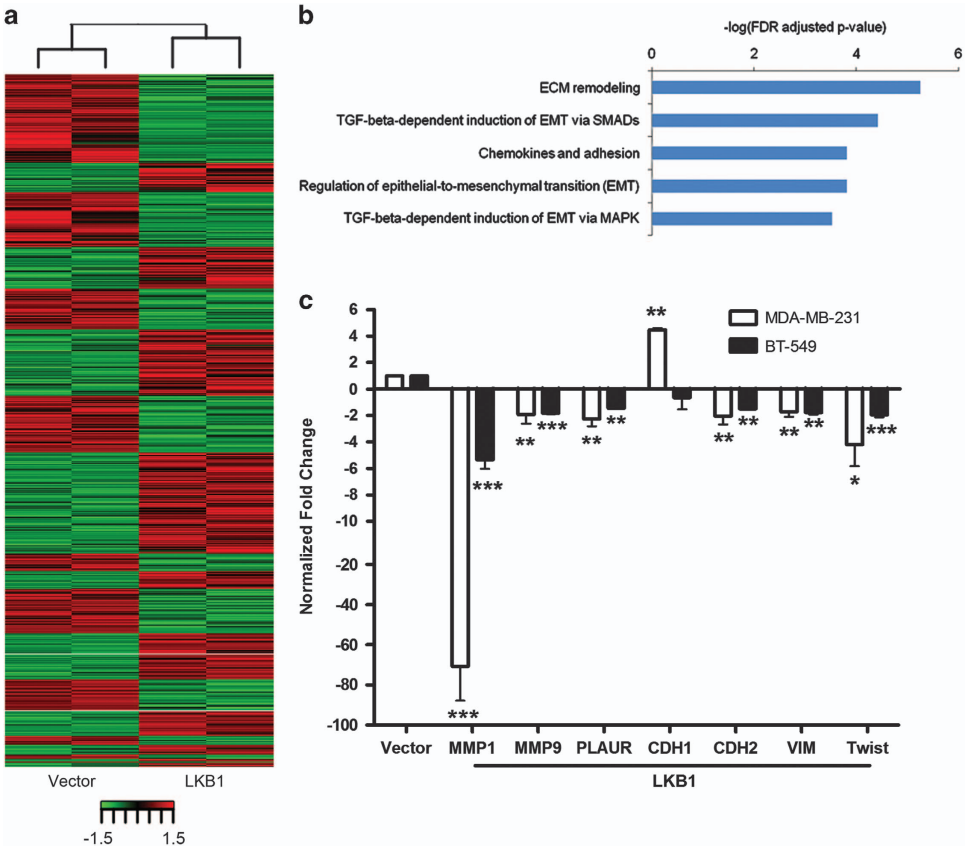

Figure 4. LKB1 expression induces anti-invasive gene expression signature in triple-negative breast cancer (TNBC). (**a**) Heatmap of differentially expressed genes from RNA-seq analysis of MDA-MD-231-vector and -LKB1 cells. (**b**) GeneGo analysis of top biological pathways altered by LKB1 expression based on differential gene expression. Bars represent –log(*P*-value) as determined by GeneGo Metacore (Thomson Reuters). (**c**) Independent quantitative real-time PCR (qPCR) seq data confirmation of a subset of genes related to ECM remodeling and EMT in MDA-MB-231 and BT-549 cells significantly altered in cells overexpressing LKB1 compared with vector control. Bars represent average fold-change ± s.e.m. of biological triplicates. $^{*}P < 0.05$; $^{**}P < 0.01$; $^{***}P < 0.001$.

LKB1 inhibits MMP-1 activity via AP-1 transcription factor regulation

MMP-1 promoter activity was examined using a luciferase reporter assay to better assess LKB1 suppression of MMP-1. MDA-MB-231-LKB1 cells transiently transfected with the wild-type, full-length MMP-1 promoter demonstrated a suppression ($P < 0.001$) of luciferase activity of >85% compared with MDA-MB-231-vector cells (Figure 5a). As MMP-1 is a known AP-1-mediated gene, and AP-1 transcription factor-related pathways were among the most downregulated pathways in the LKB1-expressing cells, we tested the AP-1 activity in our cells by AP-1 response element luciferase assay. AP-1 luciferase activity was also significantly inhibited ($P < 0.001$) in the MDA-MB-231-LKB1 cells by >75% compared with vector cells (Figure 5b).

To determine which AP-1 transcription factors were responsible for LKB1 suppression of MMP-1 promoter activity, MDA-MB-231-LKB1 cells were transiently transfected with individual AP-1 family member expression plasmids and MMP-1-luc promoter. Only the expression of c-Jun significantly relieved ($P < 0.001$) the LKB1-mediated suppression of MMP-1 promoter activity (Figure 5c). Western blot analysis confirmed a significant decrease in the level of total c-Jun expression (0.6-fold, $P < 0.05$) and a slight but not significant decrease in phospho-c-Jun levels at S73 (0.8-fold) in MDA-MB-231-LKB1 cells compared with vector control (normalized to 1; Figure 5d).

p38/MAPK signaling mediates LKB1 regulation of MMP-1

The expression levels of known upstream signaling factors of c-Jun were also examined by western blot (Figure 6a). Although no changes were observed in extracellular signal-regulated kinases 1 and 2 or c-Jun N-terminal kinase expression at either the phospho or total levels, the phosphorylation of p38/MAPK at T180/Y182 were significantly diminished (0.52-fold, $P < 0.01$) in the LKB1-expressing MDA-MB-231 cells. In addition to activating c-Jun by phosphorylation, p38/MAPK is known to regulate the transcription of c-Jun, fitting with our finding of decreased total c-Jun in the LKB1-expressing cells. The phosphorylation levels of upstream activators of p38 were also examined. MKK3/6 were decreased in the MDA-MB-231-LKB1 cells (0.4-fold, $P < 0.05$) compared with vector control (set to 1; Figure 6b), whereas no significant changes were observed in other p38 regulators tested (data not shown). Together, these data indicate that LKB1 regulation of MMP-1 is mediated through p38/MAPK signaling, at or above the level of MKK3/6 phosphorylation.

DISCUSSION

The acquired capability of invasion and metastasis by cancer cells is ultimately responsible for 90% of human cancer deaths.[22] The basal subtype of breast cancer, as classified by genomic approach,[1–3] has an aggressive clinical history as is evidenced by frequent progression to a metastatic phenotype.[1,2,7,8] Basal

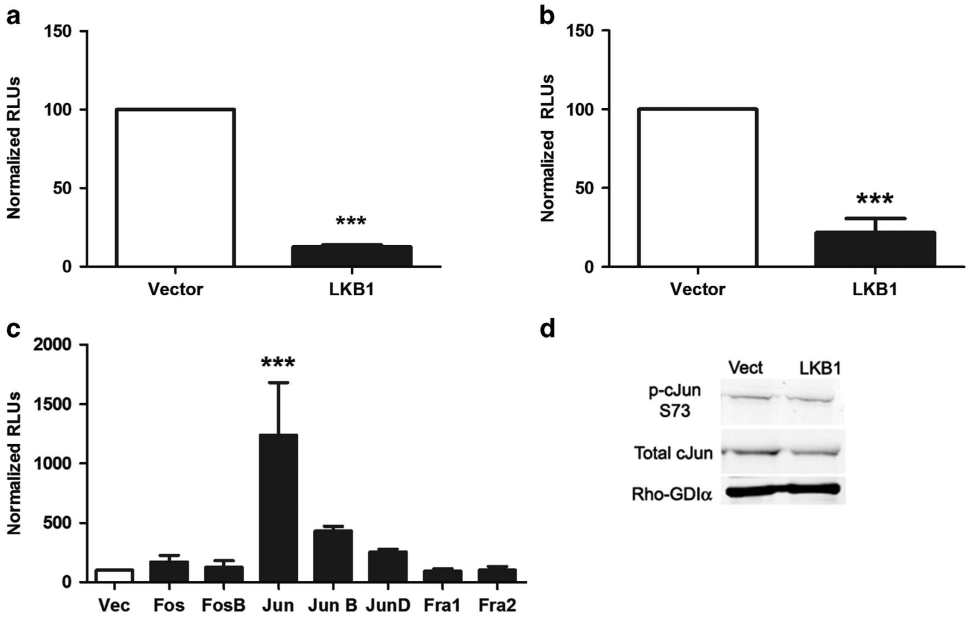

Figure 5. LKB1 expression is associated with decreased MMP-1 and AP-1 activity. (**a**) MDA-MB-231 cells were transiently transfected with LKB1 or vector control and MMP-1-luciferase plasmid for 24 h. Cells were lysed and luciferase levels measured. Bars represent relative light units (RLUs) normalized to vector control cells ± s.e.m. of triplicate experiments. (**b**) MDA-MB-231-vector or MDA-MB-231-LKB1 cells were transfected with AP-1-luciferase plasmid for 24 h. Cells were lysed and luciferase levels measured. Bars represent RLUs normalized to vector control cells ± s.e.m. of triplicate experiments. (**c**) MDA-MB-231-LKB1 cells were transiently transfected with expression plasmids for each individual AP-1 family member (Fos, FosB, Jun, JunB, JunD, Fra1, Fra2) or vector control and MMP-1-luciferase plasmid for 24 h. Cells were lysed and luciferase levels measured. Bars represent RLUs normalized to vector control cells ± s.e.m. of triplicate experiments. $^{***}P < 0.001$. (**d**) Western blot analysis of MDA-MB-231-vector or -LKB1 stable cells for phospho-(S73) and total c-Jun. Rho-GDIα serves as a loading control. Blot representative of three independent experiments.

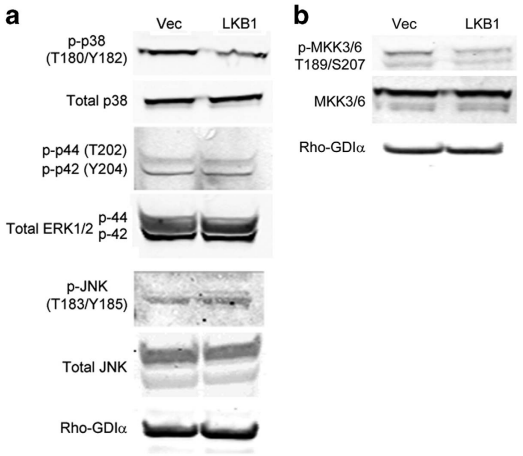

Figure 6. LKB1 expression regulates p38/MAPK signaling. Western blot analysis of MDA-MB-231-vector or -LKB1 cells for (**a**) phospho- and total p38, extracellular signal-regulated kinases 1 and 2 (ERK1/2) and c-Jun N-terminal kinase (JNK), or (**b**) phospho- and total MKK3/6. Rho-GDIα serves as a loading control. Blot representative of three independent experiments.

subtype breast cancer, preferentially negative for ER and PR and lack HER2 overexpression,[4,6] are characterized by a higher prevalence in African-American women, more frequent occurrence in younger patients, and are more aggressive than other molecular subgroups.[4,6,8,9,21] It remains critical to identify novel targets for the treatment of this breast cancer subtype.

The emerging role of the LKB1 signaling axis in carcinogenesis has suggested that LKB1 and its downstream signaling represent important tumor suppressors in multiple cancer types.[12,23,24] Although somatic mutations in LKB1 have been identified in numerous solid tumor malignancies,[14,25] more commonly observed in invasive breast carcinoma is a decreased expression of LKB1 associated with decreased RFS[13] (Figure 1), supporting its tumor-suppressor activity. In addition, loss of heterozygosity of LKB1 (19p13.3) has been documented in brain metastases arising from spontaneous cancers, including breast at a frequency of $>50\%$.[13] In addition, LOH of LKB1 was observed at much higher frequency in brain metastases compared with primary brain tumors, suggesting a role for LKB1 expression in breast cancer metastasis. Lack of frequent mutation of LKB1 in sporadic breast cancer also supports the feasibility of successful therapeutic induction of normal LKB1 signaling.

Our data, both *in vitro* and *in vivo*, demonstrate LKB1-induced suppression of not only tumorigenesis, but also the metastatic capacity of basal-type breast cancer cell systems. Objective whole transcriptomics analysis revealed suppression of ECM remodeling, EMT, cell adhesion and MAPK signaling in LKB1-expressing cells. LKB1 is known to have a role in cell polarity,[26] and it has recently been shown that LKB1, through interaction with Nischarin, can regulate migration of breast cancer cells through activation of cofilin to inhibit actin polymerization.[27] Our findings indicate an additional mechanism of LKB1 inhibition of cell invasion and metastasis of BLBC mediated, at least in part, by substantial inhibition of MMP-1 transcription via suppression of MAPK/AP-1 pathways, specifically inhibition of MKK3/6-p38 signaling activation.

Activation of LKB1 signaling represents potential for development of novel therapeutics particularly for patients exhibiting BLBC/triple-negative breast disease with low endogenous LKB1 expression. LKB1 is an upstream kinase that acts as a 'blockade' to many oncogenic pathways. For instance, LKB1 is known to regulate and/or interact with mediators of mTOR (AMPK/PRKAA1/2),[28] cAMP-dependent protein kinase (PKA, CREB and CREB co-activators),[29,30] p53,[31–33] STAT3,[34] TGF-beta (SMAD4)[35] and MYC [36] signaling pathways. In addition, LKB1 has

been indicated to have a vital role in DNA double-strand break repair.[37] Identifying interaction pathways for LKB1 allows for a unique opportunity to dissect multiple cancer-associated pathways. Novel anticancer therapeutics designed to enhance LKB1 expression and signaling, representing a common signaling node, has the potential to target multiple pro-metastatic and pro-proliferative pathways simultaneously. The broad anticancer effects of LKB1 signaling on cell metabolism, stress response, polarity, tumorigenesis and now metastasis, signify an area of great promise for novel drug development; particularly in the highly aggressive and metastatic basal subtype of breast cancer.

Conclusions

The studies shown here clearly indicate a role of LKB1 signaling in the regulation of breast cancer cell invasion and metastasis, particularly in the aggressive triple-negative subtype. Our RNA-seq data further show inhibition of several pathways involved in the EMT, ECM degradation and cellular adhesion, key regulators of the metastatic process. Specifically, MMP-1 suppression by LKB1 was mediated through p38/AP-1 signaling. Combined, these data support the development of therapeutics to activate the LKB1 pathway for the treatment of highly metastatic breast disease.

MATERIALS AND METHODS

Cell lines and reagents

Human MDA-MB-157, MDA-MB-231, BT-549 cell lines were obtained from the American Type Culture Collection (ATCC, Manassas, VA, USA) and are characterized as triple-negative/basal B mammary carcinoma.[7] MCF-7 cells, characterized as ER-positive/PgR-positive luminal mammary carcinoma, have been previously described.[20] The ER-positive/PgR-positive human breast cancer cell line MDA-MB-361 was acquired from ATCC.[38] Liquid nitrogen stocks were made upon receipt and maintained until the start of each study. Cells were used for no more than 6 months after being thawed with periodic recording of morphology and doubling times to ensure maintenance of phenotype. Cells were maintained as described previously.[21]

Kaplan–Meier online plotter analysis

Kaplan–Meier RFS curves were plotted for LKB1 expression using www.kmplot.com.[16] Using publically available gene expression data of 22 277 genes generated from Affymetrix microarrays (GEO, EGA and TCGA data sets) of 2977 breast patient samples, the PostgreSQL server integrates gene expression with patient clinical data, in this case relapse-free survival, to generate Kaplan–Meier curves, HR with 95% confidence and logrank *P*-values. Data sets for breast cancer can be filtered by hormone receptor, lymph node and grade status, as well as molecular subtype.

Western blot

Western blot analyses were conducted as previously published.[39] Membranes were probed with the following primary antibodies: phospho-LKB1 (S428), total LKB1/STK11, phospho-cJUN (S73), total cJUN/JUN, phospho-p38 (T180/Y182), total p38/MAPK14, phospho-extracellular signal-regulated kinases 1 and 2 p44 (T202) and p44 (Y204), total extracellular signal-regulated kinases 1 and 2 (MAPK3/1), phospho-c-Jun N-terminal kinase (T183/Y185), total c-Jun N-terminal kinase/MAPK8, phospho-MKK3/6 (Ser189/207), total MKK3/MAP2K3, total MKK6/MAP2K6, Rho-GDI-alpha/ARHGDIA. All primary antibodies were purchased from Cell Signaling Technology (Danvers, MA, USA) and used at a 1:1000 dilution. IR-tagged secondary antibodies were obtained from LiCor Biosciences (Lincoln, NE, USA) and used at a 1:10 000 dilution in 5% bovine serum albumin. Blots were analyzed using the Odyssey Infrared Imaging System (LiCor Biosciences). Biological triplicate experiments were conducted and representative images shown.

Stable cell generation

MDA-MB-231 and BT-549 cells were plated at approximately 50% confluence in 10% Dulbecco's modified Eagle's medium (DMEM) and allowed to adhere overnight. The following day, cells were transfected with 5 μg of pcDNA-vector or pcDNA-FLAG-LKB1 (Addgene plasmid 8590)[40] expression plasmids using Attractene (Qiagen, Germantown, MD, USA) as per the manufacturer's instructions. After 24 h, the transfection media were replaced with fresh culture media and cells treated with neomycin to select for transfected cells. Multiple independent transfections were conducted, and pooled populations confirmed by western blot following stable selection. Representative western blot is shown (Figure 2b).

Transwell migration and invasion assays

Migration assays were performed following the manufacturer's instructions (BD Biosciences, San Jose, CA, USA) and as previously published.[41] Cells were seeded at a density of 2.5×10^4 cells per well in serum-free media, with DMEM supplemented with 10% fetal bovine serum (10%) was used as a chemoattractant. DMEM without fetal bovine serum (0%) served as a negative control. After 24 h, migrated cells were fixed to the membranes and stained. Migrated cells visualized by microscopy, and data are represented as number of migrated cells per field of view ± s.e.m. for triplicate experiments.

Invasion assays were conducted in the same manner as the migration assays using Matrigel-coated (BD Biosciences) transwell membranes and a seeding density of 5×10^4 cells per well. Data are represented as number of invaded cells per field of view ± s.e.m. for triplicate experiments.

MTT cell proliferation assays

Proliferation of LKB1 expression cells and vector cells was measured by MTT (3-(4,5–dimethylthiazol–2–yl)-2,5-diphenyltetrazolium bromide) cell proliferation assays according to the manufacturer's protocol (Invitrogen, Grand Island, NY, USA) as previously published.[21] Data represented as mean percentage of corresponding vector control cell proliferation ± s.e.m. of biological triplicate experiments with internal duplicates.

Animal xenograft studies

Xenograft tumor studies were conducted as previously described.[20,21] SCID/beige female mice (4–6 weeks old) were obtained from Charles River Laboratories (Wilmington, MA, USA). The animals were allowed a period of adaptation in a sterile and pathogen-free environment with food and water *ad libitum*. In all, 5×10^6 viable MDA-MB-231-pcDNA-vector or MDA-MB-231-pcDNA-LKB1 cells mixed with reduced growth factor Matrigel were injected into each side of the inguinal mammary fat pad ($N = 5$ mice per group). Tumor size was measured with a digital caliper and volume calculated using the formula $4/3\pi LS^2$ (L = larger radius, S = smaller radius). At necropsy, animals were killed by cervical dislocation following CO_2 exposure. Tumors, livers and lungs were removed and snap frozen or fixed in 10% formalin for future analysis. All procedures involving animals were conducted in compliance with State and Federal laws, the US Department of Health and Human Services, and guidelines established by Tulane University Animal Care and Use Committee. The facilities and laboratory animals programs of Tulane University are accredited by the Association for the Assessment and Accreditation of Laboratory Animal Care.

Quantification of lung metastasis

Following formalin fixation and paraffin embedding, lungs samples were sectioned and stained with hematoxylin and eosin. Section images were captured using the Aperio ScanScope and metastasis volume calculated via Spectrum software version 10.2.2.2315 (Aperio Technologies, Vista, CA, USA).

RNA isolation and quantitative reverse transcription real-time PCR

Cells were plated in 10% DMEM at 70% confluency harvested after 24 h using a mix of phosphate-buffered saline and EDTA. Total RNA was isolated using the RNeasy kit, according to the manufacturer's instructions (Qiagen, Valencia, CA, USA). The quantity and quality of the RNA were determined by absorbance at 260 and 280 nm using the ND-1000 (NanoDrop, Wilmington, DE, USA). In all, 1 μg total RNA was reverse transcribed using the Bio-Rad First Strand cDNA synthesis kit following the manufacturer's protocol (Bio-Rad, Hercules, CA, USA), and assayed via quantitative real-time PCR to assess gene expression changes. Primers are as follows: *MMP1* F—5'-TTTCATTTCTGTTTTCTGGCCA-3'; *MMP1* R—5'-CATCTCTGTCGGCAAATTCGT-3'; *MMP9* F—5'-GAGCTGTGCGTCTTCCCCTTC-3'; *MMP9* R—5'-GGAATGATCTAAGCCCAGTGC-3'; *PLAUR* F—5'-GGTGACGCCTTCAGCATGA-3'; *PLAUR* R—5'-CCCACTGCGGTACTGGACAT-3'; *CDH1* F—5'-AGGTGACAGA

GCCTCTGGATAGA-3'; *CDH1* R—5'-TGGATGACACAGCGTGAGAGA-3'; *CDH2* F—5'-GCCCCTCAAGTGTTACCTCAA-3'; *CDH2* R—5'-AGCCGAGTGATGGTCCAATTT-3'; *VIM* F—5'-CGTCCACCCGCACCTACAGC-3'; *VIM* R—5'-GCCAGCGAGAAGTCCACCGAG-3'; *TWIST1* F—5'-TGTCCGCGTCCCACTAGC-3'; *TWIST1* R—5'-TGTCCATTTTCTCCTTCTCTGGA-3'; *Actin/ACTB* F—5'-TGAGCGCGGCTACAGCTT-3'; *Actin/ACTNB* R—5'-CCTTAATGTCACACACGATT-3'. Quantitative reverse transcriptase–PCR was conducted as previously published.[41] Data represented as normalized fold expression compared with vector control of biological triplicate samples ± s.e.m.

RNA-sequencing analysis

Read preparation, repeat masking and read mapping were conducted as previously published (see sections 2.16–2.18 in Miller *et al*.[42]). In addition to our published methods, reads were mapped using custom perl scripts. A read is considered mapped to a gene if all but 2 (or fewer) bases of the read map to annotated exons of a gene (although the exon boundaries do not have to correspond to an identified isoform). After mapping, the collapsed read set was expanded back to its original size both so that the disposition for every read could be accounted for and to facilitate the use of other tools expecting a standard Sequence Alignment/Map (SAM) formatted data set. Counts were determined from the expanded set of reads. Typically, a read was mapped to one and only one annotated element in the human genome. Although unlikely owing to the strandedness of the reads, a read that was mapped to multiple genes in the genome incremented the count for each gene. Reads that map to multiple locations were marked as ambiguous and were not counted. Reads that were marked as repetitive (owing to RepeatMasker) were used only if they could be mapped to a unique location in the genome.

Differential gene expression was determined using the edgeR software (version 2.6.0)[43] by supplying it the raw gene counts. Dispersion was estimated using both the estimateCommonDisp and estimateTagwiseDisp methods.[44,45] A prior.n value = 10 was used for running estimateTagwiseDisp. The exactTest method was run using default parameters allowing edgeR to decide which dispersions to use.[46] Pathway analysis was performed using GeneGo Metacore (Thomson Reuters, New York, NY, USA). The Enrichment Analysis Workflow was performed using the gene list, fold-change and *P*-value scores generated by edgeR. A threshold *P*-value of < 0.05, and threshold fold-change < 0.5 was set when performing the analysis in GeneGo. Raw data (fastq) and analyzed gene expression data files are available through GEO (accession number GSE56882).

Enzyme-linked immunosorbent assay for CDH1

Alterations of CDH1 protein levels in MDA-MB-231-vector or -LKB1 cells were determined by enzyme-linked immunosorbent assay as previously described[21] and according to the manufacturer's instructions (R&D Systems, Minneapolis, MN, USA). The absorbance was read at 450 nm on a Synergy HT Multi-Mode Microplate Reader (Bio-Tek, Winooski, VT, USA). Data are represented as mean pg/ml of CDH1 ± s.e.m. of duplicate experiments with internal triplicates.

Transcription factor assay

The Cignal 45-Pathway Reporter assay (SABiosciences, Valencia, CA, USA) was used per the manufacturer's instructions. MDA-MB-231-vector and -LKB1 cells were plated in the Cignal 45-pathway reporter assay 96-well plate format for reverse transfection. The following day, the medium was replaced with full growth medium and transcription factor activity determined using the Dual-Luciferase Reporter Assay System (SABiosciences).

Reporter gene assay

Luciferase reporter gene assays were conducted as previously published.[47] Briefly, cells were plated at a density of 5×10^5 cells per well in 24-well plates in 10% DMEM and allowed to adhere overnight. For basal activity experiments, cells were transfected for 24 h with pAP-1(PMA)-luciferase plasmid (Clontech, Palo Alto, CA, USA) or human collagenase (MMP-1)-luciferase (hColl-Luc) (kindly provided by Dr Lynn Matrisian, Vanderbilt University)[48] using Attractene Transfection reagent (Qiagen, Valencia, CA, USA) according to the manufacturer's protocol. For AP-1 family member transfection, cells were transfected with MMP1-luciferase plasmid and FOS, FOSB, JUN, JUNB, JUND, FRA1/FOSL1, FRA2/FOSL2 or vector plasmids for 24 h. Cells were harvested, lysed and luciferase activity measured using Luciferase Assay Substrate (Promega, Madison, WI, USA) in a Berthold AutoLumat Plus luminometer. Data represented as normalized relative light units ± s.e.m. of triplicate independent experiments with internal duplicates.

Statistical analyses

Statistical analyses were carried out with GraphPad Prism software (Graph-Pad Software, Inc., San Diego, CA, USA). Studies involving more than two groups were analyzed by one-way analysis of variance followed by Tukey's *post-hoc* multiple comparison tests. All others were subjected to unpaired Student's *t*-test, with $P < 0.05$ considered statistically significant.

CONFLICT OF INTEREST

The authors declare no conflict of interest.

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AUTHOR CONTRIBUTIONS

LVR drafted, edited, and revised the manuscript, conducted KM analysis, western blot analysis, stable cell line generation, tumor xenograft and metastasis studies, RNA-seq sample generation and analysis, and quantitative real-time PCR (qPCR) analysis, as well as participated in the concept and design of study. CRT ran MTT proliferation assays and CDH1 enzyme-linked immunosorbent assay (ELISA). VTH, HEB, and DG were involved in animal study necropsy and quantitation of metastasis. ECM was integral to RNA-seq data analysis. SE ran all luciferase and transcription factor assays. DBM, AB, DR, HT and KPN were responsible for RNA-seq sample preparation, library construction, alignment and gene expression data generation. MEB participated in the concept and design of the study, and revising and editing of the manuscript. BMC-B conceived of the study, participated in the study design and revising and editing of the manuscript. All authors have read and approved of the final manuscript.

REFERENCES

1 Perou CM, Sorlie T, Eisen MB, van de Rijn M, Jeffrey SS, Rees CA *et al.* Molecular portraits of human breast tumours. *Nature* 2000; **406**: 747–752.
2 Russnes HG, Navin N, Hicks J, Borresen-Dale AL. Insight into the heterogeneity of breast cancer through next-generation sequencing. *J Clin Invest* 2011; **121**: 3810–3818.
3 Curtis C, Shah SP, Chin SF, Turashvili G, Rueda OM, Dunning MJ *et al.* The genomic and transcriptomic architecture of 2,000 breast tumours reveals novel subgroups. *Nature* 2012; **486**: 346–352.
4 Schneider BP, Winer EP, Foulkes WD, Garber J, Perou CM, Richardson A *et al.* Triple-negative breast cancer: risk factors to potential targets. *Clin Cancer Res* 2008; **14**: 8010–8018.
5 Reis-Filho JS, Tutt AN. Triple negative tumours: a critical review. *Histopathology* 2008; **52**: 108–118.
6 Dent R, Trudeau M, Pritchard KI, Hanna WM, Kahn HK, Sawka CA *et al.* Triple-negative breast cancer: clinical features and patterns of recurrence. *Clin Cancer Res* 2007; **13**: 4429–4434.
7 Lehmann BD, Bauer JA, Chen X, Sanders ME, Chakravarthy AB, Shyr Y *et al.* Identification of human triple-negative breast cancer subtypes and preclinical models for selection of targeted therapies. *J Clin Invest* 2011; **121**: 2750–2767.
8 Toft DJ, Cryns VL. Minireview: basal-like breast cancer: from molecular profiles to targeted therapies. *Mol Endocrinol* 2011; **25**: 199–211.
9 Gelmon K, Dent R, Mackey JR, Laing K, McLeod D, Verma S. Targeting triple-negative breast cancer: optimising therapeutic outcomes. *Ann Oncol* 2012; **23**: 2223–2234.

10 Katajisto P, Vallenius T, Vaahtomeri K, Ekman N, Udd L, Tiainen M *et al.* The LKB1 tumor suppressor kinase in human disease. *Biochimica et Biophysica acta* 2007; **1775**: 63–75.
11 van Lier MG, Wagner A, Mathus-Vliegen EM, Kuipers EJ, Steyerberg EW, van Leerdam ME. High cancer risk in Peutz-Jeghers syndrome: a systematic review and surveillance recommendations. *Am J Gastroenterol* 2010; **105**: 1258–1264; author reply 65.
12 Herrmann JL, Byekova Y, Elmets CA, Athar M. Liver kinase B1 (LKB1) in the pathogenesis of epithelial cancers. *Cancer Lett* 2011; **306**: 1–9.
13 Fenton H, Carlile B, Montgomery EA, Carraway H, Herman J, Sahin F *et al.* LKB1 protein expression in human breast cancer. *Appl Immunohistochem Mol Morphol* 2006; **14**: 146–153.
14 Guldberg P, thor Straten P, Ahrenkiel V, Seremet T, Kirkin AF, Zeuthen J. Somatic mutation of the Peutz-Jeghers syndrome gene, LKB1/STK11, in malignant melanoma. *Oncogene* 1999; **18**: 1777–1780.
15 Shen Z, Wen XF, Lan F, Shen ZZ, Shao ZM. The tumor suppressor gene LKB1 is associated with prognosis in human breast carcinoma. *Clin Cancer Res* 2002; **8**: 2085–2090.
16 Gyorffy B, Lanczky A, Szallasi Z. Implementing an online tool for genome-wide validation of survival-associated biomarkers in ovarian-cancer using microarray data from 1287 patients. *Endocr Relat Cancer* 2012; **19**: 197–208.
17 Marcus AI, Zhou W. LKB1 regulated pathways in lung cancer invasion and metastasis. *J Thoracic Oncol* 2010; **5**: 1883–1886.
18 Liu W, Monahan KB, Pfefferle AD, Shimamura T, Sorrentino J, Chan KT *et al.* LKB1/STK11 inactivation leads to expansion of a prometastatic tumor subpopulation in melanoma. *Cancer Cell* 2012; **21**: 751–764.
19 Zhuang ZG, Di GH, Shen ZZ, Ding J, Shao ZM. Enhanced expression of LKB1 in breast cancer cells attenuates angiogenesis, invasion, and metastatic potential. *Mol Cancer Res* 2006; **4**: 843–849.
20 Rhodes LV, Muir SE, Elliott S, Guillot LM, Antoon JW, Penfornis P *et al.* Adult human mesenchymal stem cells enhance breast tumorigenesis and promote hormone independence. *Breast Cancer Res Treat* 2010; **121**: 293–300.
21 Tate CR, Rhodes LV, Segar HC, Driver JL, Pounder FN, Burow ME *et al.* Targeting triple-negative breast cancer cells with the histone deacetylase inhibitor panobinostat. *Breast Cancer Res* 2012; **14**: R79.
22 Hanahan D, Weinberg RA. Hallmarks of cancer: the next generation. *Cell* 2011; **144**: 646–674.
23 Gao Y, Ge G, Ji H. LKB1 in lung cancerigenesis: a serine/threonine kinase as tumor suppressor. *Protein Cell* 2011; **2**: 99–107.
24 Sahin F, Maitra A, Argani P, Sato N, Maehara N, Montgomery E *et al.* Loss of Stk11/Lkb1 expression in pancreatic and biliary neoplasms. *Mod Pathol* 2003; **16**: 686–691.
25 Wingo SN, Gallardo TD, Akbay EA, Liang MC, Contreras CM, Boren T *et al.* Somatic LKB1 mutations promote cervical cancer progression. *PloS ONE* 2009; **4**: e5137.
26 Partanen JI, Tervonen TA, Klefstrom J. Breaking the epithelial polarity barrier in cancer: the strange case of LKB1/PAR-4. *Philos Trans R Soc Lond B Biol Sci* 2013; **368**: 20130111.
27 Jain P, Baranwal S, Dong S, Struckhoff AP, Worthylake RA, Alahari SK. Integrin-binding protein nischarin interacts with tumor suppressor liver kinase B1 (LKB1) to regulate cell migration of breast epithelial cells. *J Biol Chem* 2013; **288**: 15495–15509.
28 van Veelen W, Korsse SE, van de Laar L, Peppelenbosch MP. The long and winding road to rational treatment of cancer associated with LKB1/AMPK/TSC/mTORC1 signaling. *Oncogene* 2011; **30**: 2289–2303.
29 Gu Y, Lin S, Li JL, Nakagawa H, Chen Z, Jin B *et al.* Altered LKB1/CREB-regulated transcription co-activator (CRTC) signaling axis promotes esophageal cancer cell migration and invasion. *Oncogene* 2012; **31**: 469–479.
30 Komiya T, Coxon A, Park Y, Chen WD, Zajac-Kaye M, Meltzer P *et al.* Enhanced activity of the CREB co-activator Crtc1 in LKB1 null lung cancer. *Oncogene* 2010; **29**: 1672–1680.
31 Yao M, Wang Y, Shen H, Ning W, Tang J, Wang X *et al.* Identification of the core promoter of STK11 gene and its transcriptional regulation by p53. *Prog Nat Sci* 2008; **18**: 273–280.
32 Hou X, Liu JE, Liu W, Liu CY, Liu ZY, Sun ZY. A new role of NUAK1: directly phosphorylating p53 and regulating cell proliferation. *Oncogene* 2011; **30**: 2933–2942.
33 Liu L, Du X, Nie J. A novel *de novo* mutation in LKB1 gene in a Chinese Peutz Jeghers syndrome patient significantly diminished p53 activity. *Clin Res Hepatol Gastroenterol* 2011; **35**: 221–226.
34 Kim DW, Chung HK, Park KC, Hwang JH, Jo YS, Chung J *et al.* Tumor suppressor LKB1 inhibits activation of signal transducer and activator of transcription 3 (STAT3) by thyroid oncogenic tyrosine kinase rearranged in transformation (RET)/papillary thyroid carcinoma (PTC). *Mol Endocrinol* 2007; **21**: 3039–3049.
35 Moren A, Raja E, Heldin CH, Moustakas A. Negative regulation of TGFbeta signaling by the kinase LKB1 and the scaffolding protein LIP1. *J Biol Chem* 2011; **286**: 341–353.
36 Liang X, Nan KJ, Li ZL, Xu QZ. Overexpression of the LKB1 gene inhibits lung carcinoma cell proliferation partly through degradation of c-myc protein. *Oncol Rep* 2009; **21**: 925–931.
37 Ui A, Ogiwara H, Nakajima S, Kanno S, Watanabe R, Harata M *et al.* Possible involvement of LKB1-AMPK signaling in non-homologous end joining. *Oncogene* 2013; **33**: 1640–1648.
38 Rhodes LV, Bratton MR, Zhu Y, Tilghman SL, Muir SE, Salvo VA *et al.* Effects of SDF-1-CXCR4 signaling on microRNA expression and tumorigenesis in estrogen receptor-alpha (ER-alpha)-positive breast cancer cells. *Exp Cell Res* 2011; **317**: 2573–2581.
39 Martin EC, Elliott S, Rhodes LV, Antoon JW, Fewell C, Zhu Y *et al.* Preferential star strand biogenesis of pre-miR-24-2 targets PKC-alpha and suppresses cell survival in MCF-7 breast cancer cells. *Mol Carcinog* 2014; **53**: 38–48.
40 Shaw RJ, Kosmatka M, Bardeesy N, Hurley RL, Witters LA, DePinho RA *et al.* The tumor suppressor LKB1 kinase directly activates AMP-activated kinase and regulates apoptosis in response to energy stress. *Proc Natl Acad Sci USA* 2004; **101**: 3329–3335.
41 Rhodes LV, Antoon JW, Muir SE, Elliott S, Beckman BS, Burow ME. Effects of human mesenchymal stem cells on ER-positive human breast carcinoma cells mediated through ER-SDF-1/CXCR4 crosstalk. *Mol Cancer* 2010; **9**: 295.
42 Miller DF, Yan PS, Buechlein A, Rodriguez BA, Yilmaz AS, Goel S *et al.* A new method for stranded whole transcriptome RNA-seq. *Methods* 2013; **63**: 126–134.
43 Robinson MD, McCarthy DJ, Smyth GK. edgeR: a Bioconductor package for differential expression analysis of digital gene expression data. *Bioinformatics* 2010; **26**: 139–140.
44 Robinson MD, Smyth GK. Moderated statistical tests for assessing differences in tag abundance. *Bioinformatics* 2007; **23**: 2881–2887.
45 Robinson MD, Smyth GK. Small-sample estimation of negative binomial dispersion, with applications to SAGE data. *Biostatistics* 2008; **9**: 321–332.
46 McCarthy DJ, Chen Y, Smyth GK. Differential expression analysis of multifactor RNA-Seq experiments with respect to biological variation. *Nucleic Acids Res* 2012; **40**: 4288–4297.
47 Frigo DE, Tang Y, Beckman BS, Scandurro AB, Alam J, Burow ME *et al.* Mechanism of AP-1-mediated gene expression by select organochlorines through the p38 MAPK pathway. *Carcinogenesis* 2004; **25**: 249–261.
48 Gaire M, Barro CD, Kerr LD, Carlisle F, Matrisian LM. Protein kinase C isotypes required for phorbol-ester induction of stromelysin-1 in rat fibroblasts. *Mol Carcinogenesis* 1996; **15**: 124–133.

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